Hymenolepis diminuta: The pathophysiology of infection in the intermediate host, *Tenebrio molitor*.

Hilary Hurd

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ABSTRACT

In T. molitor infected with metacestodes of H. diminuta total haemolymph free protein concentration, determined by the Lowry method, ranged from 62 to 81 mg/ml in females and was significantly lower in males (36-54mg/ml). A 47% increase was detected in female beetles 12 days or more post-infection, no such difference being detected at an earlier stage, nor in males at any age examined. Using SDS PAGE, 13 bands were separated from haemolymph, bands 2/3 and 7/8 being present in greater concentrations in female beetles. Molecular weight determinations and histochemical evidence suggested that these proteins were vitellogenins. Densitometric analysis revealed that these bands alone were elevated in haemolymph from females 12 days post-infection, no such elevation being detected at an earlier stage, nor in infected male beetles. Infection did not affect fat body wet-weight nor protein content. However, in vitro culture of fat bodies with ^{14}C-leucine revealed a 61% decrease in protein synthesis to be associated with infection. In vivo sequestration of labelled proteins by ovaries from infected beetles was significantly decreased, the majority of the label being detected in the vitellogenic fraction of ovary homogenates. Various parameters of fecundity were measured over a period of 30 days; total egg-volume and egg protein content were unaffected by infection. However, the second peak of egg laying was delayed, egg viability decreased and total protein content of eggs reduced in infected females. Sixteen haemolymph free amino acids were detected, total concentrations ranging from 34-94 mM. Although overall concentrations were not affected by infection, significant differenes occurred in individual amino acids, these being most marked in female beetles. The literature concerning host parasite interactions has been reviewed and it is suggested that the above pathological effects may be due to an interference by the parasite with the host endocrine system.

CONTENTS

All chemicals used were "Analar" or the best available grade obtainable from Sigma Chemical Company Ltd. unless otherwise stated.

Statistical analysis of data was performed on a GEC "A" machine (Keele University Computer Centre) with the exception of student "t" tests, used to test for significance. Word processing facilities were used for document production.

Chapter 1

INTRODUCTION

Insects act as intermediate hosts for the metacestodes of several Cyclophyllidea, including the rat-tapeworm, Hymenolepis diminuta. The life cycle of this worm is short and the parasite can easily be maintained in hosts that are common laboratory animals viz the rat and the mealworm beetle. Thus it provides a useful model system and, as such, has received much attention from parasitologists. Studies have, however, been directed almost exclusively towards the adult stage in the vertebrate definitive host, and our knowledge of the biology of the cysticercoid in the insect host is by comparison very limited. The work presented in this thesis is a contribution to redressing this imbalance by focussing attention upon the pathophysiology of the invertebrate host, Tenebrio molitor.

It is evident from an examination of the literature concerning insect-metazoan parasite relationships that, with the exception of the parasitoids, there is little evidence of gross pathology in natural hosts and that parasitism need not be synonymous with overt pathogenicity. Some degree of host response to infection has, however, been reported in the majority of cases examined and can be assessed in terms of a change in for example, morphology, histology, physiology, behavioural patterns or population dynamics. Recent reviews by Von Brand (1979), Thompson (1983a) and Freeman (1983) have discussed various aspects of the pathophysiology of infection in invertebrate hosts and it is clear that, whilst some attention has been directed to the areas of nematode-insect associations, there is a paucity of information on cestode-insect relationships.

The majority of metazoan parasites of insects inhabit the

nutrient-rich environment of the haemocoel where they have access to metabolites necessary for growth and development. The host response to the withdrawal of nutrients by an endoparasite could take the form of an increase in food intake or of a depletion of food reserves and/or haemolymph metabolites. The nature of haemolymph as a dynamic tissue has been discussed by Jungreis (1980), and it is clear from the examples quoted below that insects are capable of responding in a variety of ways to the competition for blood metabolites that must exist between the symbiotes. There is a similarity between the response to parasitic infection and that of host starvation. However the view that host pathogenicity is a direct result of competition for food was dismissed by Von Brand (1979). He maintained that, with notable exceptions, parasite biomass is small and that food withdrawal would be insignificant compared with the total available metabolites. Thompson (1983) considered that host pathogenicity is often the result of parasite-induced changes in metabolic and developmental patterns, indicative of a nighly integrative relationship. The examples of host-parasite interactions discussed below will serve to illustrate the broad spectrum of responses that insect hosts are capable of making.

The uptake of low molecular-weight nutrients, such as amino acids, by insect parasites has been demonstrated by several authors. Gordon & Webster, (1972) showed that the varied rate of *in vitro* amino acid incorporation into proteins by *Mermis nigrescens* corresponded to its *in vivo* growth pattern. This amino acid absorbance was confirmed by Rutherford, Webster & Barlow (1977), who concluded that the cuticular uptake was sodium and energy independent. Gordon & Webster (1971) showed that amino acid concentrations in the haemolymph of *Schistocerca gregaria* were unaffected by *M. nigrescens*, and similar results have been described

in other nematode-insect associations (Schmidt & Platzer, 1980a and Thong & Webster, 1975a). Lackie (1972) was also unable to detect a change in total haemolymph free amino acids in the cockroach, Periplaneta americana, infected with the acanthocephalan Moniliformis dubius. In contrast, many accounts of the effect of parasites upon host haemolymph amino acids describe a change affecting individual amino acids, although the total concentrations remain stable. The protozoan-insect associations described by Mack, Samuels & Vanderberg (1979) for Plasmodium berghei infections of Anopheles stephensi and by Wang & Moeller (1970) for Nosema-infected female honey bees are examples of this. Gordon, Condon, Edgar & Babie (1978) found both qualitative changes and an overall depletion in haemolymph amino acids in the mermithid-parasitized simuliid, Prosimulium mixtum-fuscum, and suggested that the host was in a state of physiological starvation. In the summer-developing blackfly Simulium venustum, harbouring the same parasite, by contrast, fewer amino compounds were depleted and many increased in concentration. Ormerod (1967), investigating infections of Rhodnius prolixus with Trypanosoma rangeli, suggested that the large increase in the amount of some amino acids in insects infected with a pathogenic strain was connected with an immune response on behalf of the host.

The role of amino acids as osmotically active constituents of haemolymph was stressed by Sutcliffe (1963). In view of this it would presumably be advantageous for the insect to preserve the total concentration of haemolymph free amino acids by compensating in some way for the drain on these metabolites caused by haemocoel parasites. Many authors have suggested that this homeostasis is maintained at the expense of host proteins and an extensive literature exists concerning the depletion of host proteins by nematodes. For example, Thong & Webster (1975a) reported a decrease in haemolymph proteins in

the female beetle Dendroctonus pseudotsugae infected with Contortylenchus reversus and severe protein depletion was also detected in three mosquitoe species infected with mermithids (Womersley & Platzer, 1982). Lieutier (1984) reported that two nematodes caused fat body proteins to be lowered in the beetle Ips sexdentalis; Parasitaphelenchus species also caused ovarian protein levels to decrease, although Contortylenchus diplogaster had no such effect. Mermithid infections of larval blackflies were also found to result in haemolymph protein depletion, accompanied by fat body tissue autolysis and depletion of glycogen reserves and blood glucose, although trehalose concentrations were unaffected (Gordon, Condon, Edgar & Babie, 1978). Schmidt & Platzer (1980a) attempted to attribute the reduction in host proteins, associated with nematode infection, to the secretion by the parasite of a proteolytic enzyme. They failed, however, to find any proteins of parasite origin within the host, nor did they detect an elevation in haemolymph protease activity. Gordon, Webster & Hislop (1973), investigating mermithid parasitism in the desert locust, found that a depletion of haemolymph proteins and their subsequent replenishment one week later mirrored conditions occuring in the fat body seven days earlier. They discussed the possibility of parasite manipulation of the host endocrine system and, indeed, many of the authors quoted above suggested that alterations in host protein metabolism could be brought about either by nutritional stress resulting from lack of amino acid precursors or as a result of host hormonal changes. However, Chambers, Hitt & Hall (1975), investigating the effects of trematode infections on aquatic insects, and Andreadis & Hall (1976) examining the encapsulation of a nematode by Aedes aegypti, both linked a haemolymph free amino acid decrease in the former, and increase in the latter, to the functioning of the host defense

mechanism.

The effect of metazoan parasites upon other insect host metabolites such as carbohydrates and lipids are not so well documented. On first examination the reduction of total body lipid in Hyposoter exiguae, infected with the parasitoid Trichoplusia ni, was thought to resemble the effects of starvation (Thompson, 1982a) and the size of the parasite compared to host is such that some nutritional stress must presumably exist. However, further research showed that the fatty acid composition of neutral lipids in the larvae varied; starvation resulted in lower proportions of palmitic and oleic acids and higher levels of linoleic and linolenic acids, whereas the relative proportions of both palmitic and oleic acids increased with age in infected larvae. This suggested that some active synthesis occurred in parasitized insects, whereas nutrient deprived larvae utilized lipids as an energy source (Thompson, 1983b). Mermithid infections have been found to cause fat body tissue depletion in blackflies (Condon & Gordon, 1977), and a reduction in fat body and flight muscle lipids in Locusta migratoria (Jutsum & Goldsworthy, 1974) and Gordon, Finney, Condon & Rusted (1979) found that the overall lipid concentration of the blood of Aedes aegypti was unaltered but noted an increase in the myristic:palmitic acid ratio in the free fatty acid fraction.

The effect of parasites upon carbohydrate reserves is varied. An increase in both haemolymph non-glycogen carbohydrates and fat body glycogen in ichneumonoid infections of Trichoplusia ni was reported by Thompson (1982a), whereas a depletion in haemolymph carbohydrates was detected in mosquitoes infected with Plasmodium sp. (Mack, Samuels & Vanderburg, 1979). Trehalose concentrations were unaffected in Contortylenchus reversus infections of Dendroctonus pseudosugae (Thong & Webster, 1975a). Gordon & Webster (1971) found

that food consumption and blood volume were unaffected by Mermis nigrescens infection of Schistocerca gregaria. However blood carbohydrates were severely depleted and Gordon, Webster & Mead (1971) determined that fat body glycogen and non-glycogen carbohydrates were depleted in locusts 2-3 weeks after Mermis infections. Fat body active phosphorylases were reduced in concentration 2 weeks post-infection and inactive phosphorylases also declined by week 3. It was thus suggested that the parasite was able, directly or indirectly, to suppress glycolysis, interference with host hyperglycaemic factor being given as a possible explanation. A reduction in haemolymph carbohydrates has also been recorded for nematode infections of Culex pipiens (Schmidt & Platzer, 1980b) and Locusta migratoria (Jutsum, Agarwal & Goldsworthy, 1975).

An investigation into haemolymph carbohydrates in T. molitor infected with H. diminuta metacestodes has revealed no significant difference in trehalose concentrations. However, glucose, which is undetectable using gas-liquid chromatography in non-infected beetles, appears in trace amounts in females 6 days post-infection and is present at concentrations of 0.8 mM in females and 0.6 mM in males 9 days post-infection (Phillips, personal communication).

Attention is increasingly being directed towards the significance of host metabolic disturbances, of the type outlined above, and to the interpretation of the host parasite relationship in terms of an integrative association rather than one based purely on competition for food. Tentative forays into the realms of host endocrinology are being made by parasitologists searching for a link between infection and host pathophysiology. A sample of these are discussed below and the subject will be dealt with in more detail in Ch. 11.

Symbiotic associations involving endocrine interactions can

result in either partner being affected, thus, the host hormones may alter parasite development or the parasite may affect the host endocrine system. The synchronization of parasite-host life cycles is thought to be a result of parasite sensitivity to host hormones, changes in concentration of one or more of these acting as a cue to activate parasite resting stages (Baronio & Sehnal, 1980). It has been suggested that nematodes as well as insect parasites respond to host hormones. Davey & Hominick, (1973) have demonstrated that juvenile hormone and ecdysone affect the growth and development of nematodes *in vitro* although no direct hormonal interaction has been shown to occur *in vivo*. Stoffolano (1967) described the arrest in egg development of *Heterotylenchus autumnalis* when its host, *Musca autumnalis* entered diapause and its subsequent development when diapause was broken by activation of the corpora allata.

The interference of parasites with host moulting is well documented, for example, parasitism of *Trichoplusia ni* by the hymenopteran *Chelonus curvimaculatus* resulted in premature host pupation (Jones, Jones & Hammock, 1981) whereas its larval stadium was lengthened and pupation inhibited by the ichneumonid, *Hyposeter exiguae* (Thompson, 1982b). An alteration in neurosecretory cell or corpora allata activity has been reported in various infected insects (Strambi & Strambi, 1973; Girardie & Girardie, 1977 & Condon & Gordon, 1977) (see Ch. 11) although in most cases it has not been determined whether the effects on host endocrine systems are direct or indirect.

A reduction or elimination of host reproductive potential, or parasitic castration, is a phenomenon observed in both nematode-insect and insect-insect associations. Thong & Webster (1975b), investigating the effects of *Contortylenchus reversus* upon the Douglas Fir Beetle, *Dendroctonus pseudotsugae* observed a

reduction in the length of the primary egg galleries built by infected females and a reduction in the number of eggs laid, although egg viability was unaffected. They also recorded a 20% reduction in the size of terminal oocytes in infected beetles (Thong & Webster, 1975b) and attributed this either to the depletion of haemolymph protein or to an interference with host hormonal control of vitellogenesis. These hypotheses were not, however, investigated further. Nematode-coleopteran associations have also been reported to result in delayed oocyte maturation (Lieutier, 1982) and alteration in reproductive behaviour accompanied by female castration (Ashraf & Berryman, 1970). Mermithid parasitism of the desert locust, S. gregaria resulted in complete suppression of vitellogenesis and the resorption of terminal and penultimate oocytes by the third week of infection (Gordon et. al, 1973). This was ascribed, by the authors, to an impairment of the ability of the oocyte follicular cells to sequester proteins rather than a reduction in available vitellogenic proteins resulting from the alterations in fat body protein turnover described above. A possible relationship between parasite and host endocrine system was suggested.

In many cases the involvement of the host endocrine system in parasitic castration is largely speculative. However, more direct evidence is available with respect to the parasitization of Anacridium aegyptium by the dipteran parasitoid, Metacemyia calloti. Girardie & Girardie (1977c) found the activity of the median neurosecretory cells (M-NSC) to be impaired in parasitized females and that electrical stimulation of the pars intercerebralis increased activity and enhanced ovarian development. The depletion of haemolymph proteins, including a vitellogenic fraction, associated with this symbiosis was related to the hypoactivity of the M-NSC, which was also thought to be the cause of the inability of oocytes to

sequester protein (Giradie, 1977). The examples of host pathogenicity cited above, drawn as they are almost exclusively from nematode-insect and insect-insect associations, are a reflection of the narrow approach to research into metazoan parasite-insect relationships to date. This has perhaps been engendered by the prospect of the use of parasites from these groups as biological control agents (Platzer, 1981). Our scant knowledge of cestode-insect relationships is largely epidemiological (Keymer, 1980; 1981 & 1982) and will be discussed in Ch. 9. Literature concerning the invertebrate defense response to metazoan parasites is summarised by Freeman (1983) and it has been suggested (Lackie, 1976) that hymenolepid metacestodes either possess a surface compatible with host tissue or are able to inhibit haemocyte attachment and subsequent encapsulation. Descriptions of insect-host pathology resultant upon H. diminuta infection are confined to those of: Phillips (loc. cit) on haemolymph glucose concentrations, Granath (1980), who described an increase in water loss in T. molitor as the density of H. diminuta cysticercoids increased when maintained at 15^{o}C and desiccated for 72h, and the observations by Soltice, Arai & Scheinberg (1971) that oxygen consumption increased with infection in Tribolium confusum and T. castaneum harbouring seven and a half day old H. diminuta metacestodes.

The investigations reported in this thesis concentrated initially upon variations in the concentration of T. molitor haemolymph proteins at various times post-infection with H. diminuta metacestodes. The findings were quite unlike those reported above and led to an examination of the effect of parasitization upon host protein metabolism, haemolymph free amino acid concentrations, vitellogenesis and fecundity. The results have stimulated discussion of the possible manipulation , directly or indirectly, of the beetle

endocrine system by the tapeworm.

Chapter 2

MAINTENANCE OF PARASITE AND INTERMEDIATE HOST

Both the rat tapeworm Hymenolepis diminuta and its hosts, Tenebrio molitor and Wistar rats, have been maintained in the laboratory to provide a readily available supply of experimental animals. An account of procedures undertaken for their maintenance will follow a brief description of the life history of parasite and host.

THE LIFE CYCLE OF H.DIMINUTA

A number of rodents are able to act as definitive hosts, and reports of infections in several other mammal-species, including man, exist.

Following ingestion by a rat, activation and excystment take place in the stomach and small intestine. A number of enzymes and bile have been implicated in this process (Rothman, 1959; Caley, 1974). Once excystment has occurred, rapid growth in the small intestine of the final host follows an exponential pattern for approximately 10 days. Increases in both length and weight are influenced by several factors including host diet, host sex, the presence of other parasite species and the "crowding effect", which describes an inverse relationship between the size of mature worms and the number harboured (Kennedy 1983).

The monoecious adult produces numerous ova and spermatozoa. After fertilization the eggs pass into the uterus where they undergo embryogenesis to produce a hexacanth larva or oncosphere. The presence of shelled embryos in a gravid proglottis can be seen 9-12 days after infection. Mature proglottides are shed, disintegrate and

release their contents into the intestine, eggs pass out of the host with the faeces and are first detected 16-17 days post-infection.

The oncospheres of cyclophyllidean cestodes are surrounded by a series of envelopes that have been described by various authors (Lethbridge, 1980; Ubelaker, 1983). Reduced to their simplest these consist of an egg-shell, a sub-shell membrane, a cytoplasmic layer, the embryophore and the oncospheral membrane.

The egg-shell, unlike those of the pseudophyllidean cestodes, is not derived from a vitelline cell containing a phenoloxidase system and is thus untanned. It is, however, able to protect the oncosphere against bacterial attack and is unaffected both by proteolytic enzymes and organic solvents which disrupt weak bonds. Moczoń (1972) suggested that the hardening of the shell is due to the oxidation of the free sulphydryl groups of proteins to form S-S linkages in a pre-keratin formation. Lethbridge (1980), however, suggested that the presence of aromatic and heterocyclic amino acid residues, which may be cross-linked covalently to protein macromolecules, would explain the maintenance of the integrity of the egg-shell.

The sub-shell membrane is a mucopolysaccharide protein complex (Moczoń, 1972), which is impermeable to chemicals and protects the oncosphere against external variations in pH and osmolarity. It is unaffected by proteases, carbohydrases and lipases and may act as a barrier to prevent premature hatching; the closely applied egg-shell also provides mechanical support.

Filling the space between the egg-shell and embryophore is the cytoplasmic layer. Lethbridge (1971a) has suggested that this region, which exists in a dehydrated state, is composed of mucopolysaccharides or mucoproteins. Once the outer membranes are damaged, the cytoplasmic layer swells, facilitating the escape of the oncosphere.

Unlike the embryophore of taeniids, that of H. diminuta is not keratinized and thus is susceptible to digestion by proteolytic enzymes (Lethbridge, 1980). The composition of the oncospheral membrane is unknown but it may be a lipoprotein complex. In the mature oncosphere is found an extensive musculature, three pairs of hooks with sharply curved tips, paired penetration glands and a syncytial epithelium.

Once released from the definitive host, eggs remain viable in the faeces for approximately six months (Smyth, 1976), their future development being initiated upon ingestion by certain insects, notably cereal-feeding Coleoptera. Hatching takes place in three phases: (i) physical rupture of the egg-shell and sub-shell membrane, (ii) activation of the embryo before its release from the remaining envelopes and (iii) the enzymatic destruction of the inner envelopes and embryophore to facilitate the escape of the oncosphere.

The mandibles of the beetle puncture both the egg-shell and sub-shell membrane. Host digestive enzymes have no effect upon these envelopes and intact eggs can pass through the insect gut and remain viable. Indeed, Lethbridge (1971b) showed that 50% of ingested eggs pass through the gut of T. molitor unaltered.

Activation can be triggered by tap water, although Berntzen & Voge (1965) reported that more sustained activity of the oncosphere requires a salt solution containing $NaHCO_3$. Swelling of the gelatinous capsule surrounding the embryophore immediately follows rupture of the shell and sub-shell membrane and may assist in the expulsion of the embryophore from the shell.

Data concerning the action of enzymes upon the remaining membranes are controversial. Holmes & Fairweather (1982) maintained that hatching can be achieved without the addition of external enzymes. They suggested that secretions from the penetration glands

were extruded due to the rhythmic movements of the oncosphere and that these, combined with the hook movements, were sufficient to rupture the embryophore and tear through the cytoplasmic layer. Lethbridge (1980), on the other hand, maintained that liberation of the hexacanth is essentially a passive affair, brought about for the most part by physico-chemical factors in the beetle gut, and that lytic secretions from the penetration gland do not hasten the process. In support of this view, the addition of enzymes has been found greatly to accelerate the release of the embryo. Berntzen & Voge (1965) obtained a 100% hatch in 10-15 min when eggs were incubated in a combination of bacterial α-amylase and trypsin. Using a solution of α-amylase alone they reported that the embryophore was digested but that the gelatinous capsule remained intact. Lethbridge (1972) has pointed out that the embryophore contains no polysaccharide. He suggested that the bacterial α-amylase they used contained proteolytic contaminants and it was these that were acting on the embryophore. Berntzen & Voge (1965), however, obtained no digestion of the embryophore when an α-amylase inhibitor was added to their incubation medium. Electrophoretic separation of T. molitor mid-gut extracts revealed two proteolytic components which act on the envelopes, one destroying the embryophore, the other acting on the cytoplasmic layer and weakening the embryophore (Lethbridge, 1972). The available evidence thus suggests the presence in vivo of a dual or multi-enzyme system.

Once released from its surrounding envelopes, the motile hexacanth exhibits "breast-stroke" movements using its three pairs of hooks. These movements aid in the rapid penetration of the gut and Lethbridge (1971b) reported these to be completed within 75 min of egg ingestion. The mid-gut of T. molitor consists of a columnar epithelium with an underlying layer of loosely arranged muscle

fibres. In the adult, some of these columnar cells project through the muscle layer into the haemocoel, forming papillae, and Lethbridge (1971b) found that the majority of oncospheres migrated through these papillae, thus avoiding the musculature. He attributed the failure of the oncospheres to penetrate the larval midgut to the absence of these papillary projections. In contrast, Moczoń (1977) reported that most oncospheres were able to penetrate the adult muscle layer. He suggested that the chemical composition of the larval and adult musculature differed, larval muscle being insusceptible to penetration gland secretion, this therefore accounting for the inability of oncospheres to infect larval T. molitor. The role of the penetration gland secretion is not fully understood. It appears to have a lytic function, its mucoid content may facilitate adhesion to the gut mucosa, serve to lubricate the passage of the oncosphere through the gut and protect it against host digestive enzymes.

Metamorphosis of the oncosphere to infective metacestode occurs in the haemocoel, taking 8-10 days at 26° C. This development involves loss of penetration glands and hook musculature, although vestigial hooks persist. The development of protonephridia and nervous system takes place, together with modifications of the epithelial surface. Stages in the development of H. diminuta metacestodes have been described using light microscopy by Voge & Heyneman (1957) and at the ultrastructural level by Richards & Arme (1983). These stages are as follows: (i) development of an ovoid body which already possesess a typical metacestode tegument, (ii) growth and development of a primary cavity or lacuna, (iii) increase in the size of the lacuna, which becomes surrounded by fibroblastic cells, delineation of three body regions, fore-body containing presumptive scolex, mid-body, and cercomer, (iv) withdrawl of the presumptive scolex, this occuring 7 days post-infection at 26° C,

(Richards & Arme, 1983) and (v) histogenesis of the wall of the capsule. A change in the surface of the presumptive adult scolex, from one bearing microvilli to one bearing microtriches, begins before scolex retraction and is completed 3 days post-retraction, the age at which metacestodes are first infective to the final host.

The tapeworm life cycle is completed when infective metacestodes (still contained within their intermediate host) are ingested by the final host.

THE LIFE CYCLE OF T.MOLITOR

T. molitor exhibits a life cycle typical of holometabolous insects, the length of each stage varying according to temperature. Details of the life history of this beetle were recorded by Cotton & St. George (1929). They found that, in the wild, the larvae overwinter, one generation of insects being produced each year. Milky-white, opaque eggs, 1.75-1.80 mm in length, were laid singly or in clusters and covered with a sticky secretion that caused them to adhere to adjacent surfaces. Incubation periods ranging from 4-19 days were observed, this time increasing with decreasing temperature.

Newly emerged larvae were white, but quickly acquired a yellowish-brown colour, this colour change following each successive moult. The number of moults varied greatly, ranging from 9-20 with a mean value of 18.

Connat (1983), investigating juvenile hormone esterase activity during the last larval and pupal stages of T. molitor, detected only one peak occuring at the beginning of the pupal stage. Holometabolous insects usually exhibit two peaks of juvenile esterase activity, the first appearing during the last larval instar and triggering the larval-pupal transformation by reducing the

concentration of circulating juvenile hormone. Connat (1983) suggested that the lack of a juvenile hormone clearing mechanism at this stage could explain the variable number of larval moults.

Cotton & St. George (1929) also reported that the length of the pupal stage was dependent upon temperature, varying from 6-18 days. They also observed that the average life-span of the adult was two months. Mating occurred a few days after emergence and oviposition commenced within 5-18 days with both the frequency of oviposition and the number of eggs laid being very variable. This observation was supported by Ullmann (1973) and Gerber (1975), who both attributed these variations in fecundity to, a variation in the number of ovarioles per ovary, the asynchronous development of oocytes in adjacent ovarioles and the retention of up to 21 eggs within the oviducts of mated females. Gerber (1975) calculated that the completion of one gonadotrophic cycle took six days and suggested that the initiation of a second cycle was suppressed in virgin females.

THE MAINTENANCE OF H.DIMINUTA AND T.MOLITOR

1. Infection of the rat

Young male Wistar rats, 80-100g in weight, were infected with mature metacestodes by the following method. Beetles, at least 10 days post-infection, were decapitated and the last two abdominal segments removed. Cysticercoids were flushed out using Tyrode's saline (see Appendix 7) and grouped into batches of 30. These were picked up in a Pasteur pipette and introduced into the throat of a lightly anaesthetized rat. Once recovered, the rats were returned to the stock colony where they were kept at 19-20°C in a 12h light 12h dark cycle, with water and food (Labsure PRD) available ad libitum.

2. Collection of tapeworm eggs

Mature proglottides of H. diminuta are not passed intact but disintegrate in the intestine, releasing eggs that were separated from faeces using a salt flotation method. The litter of cages housing infected rats was collected weekly and passed through a 5 mm mesh sieve to separate bedding from faecal pellets. The faeces were soaked for 48h in tap water to disrupt the pellets and the resultant suspension was passed through a 1 mm sieve to remove coarse material. Sodium chloride was then added to make a saturated solution that was centrifuged at 1000 g for 15 min. Tapeworm eggs were sucked from the surface of the supernatant with a 25 ml syringe, washed twice in 1 litre tap water, and stored at 4° C for up to 6 months.

3. Infection of the intermediate host

Attempts to infect newly emerged beetles with H. diminuta proved unsuccessful. The cuticle of adults takes 24-48 h to complete tanning, as indicated by its hardening and darkening. It was considered that the jaws of the beetle may not be sufficiently hard to break egg-shells during the first 2 days post-emergence and thus , although the eggs were consumed, the oncosphere was unable to hatch and infect the host. For this reason beetles were routinely starved for 2 days post-emergence and infected on day 3. Throughout this study the age of the metacestode is therefore 3 days less than that of the adult beetle; for example, 15 day-old beetles harbour 12 day-old cysticercoids.

The purified egg suspension was filtered through a Whatman No.4 filter paper, and the eggs were thoroughly mixed with approximately twice their volume of apple pulp. Beetles, previously sexed (see below) and starved, were housed individually or in pairs in paper-lined plastic Petri dishes. Apple/egg mixture was placed in

the dishes between 9-10 am and left for 24 h after which remaining material was removed and the bottom of the dishes covered with bran. Insects used as controls were treated in a similar way, but given apple pulp alone. All beetles were maintained in the dark at 26° C in an atmosphere of high humidity maintained by bubbling air through water.

4. Sexing of pupae and adult T. molitor

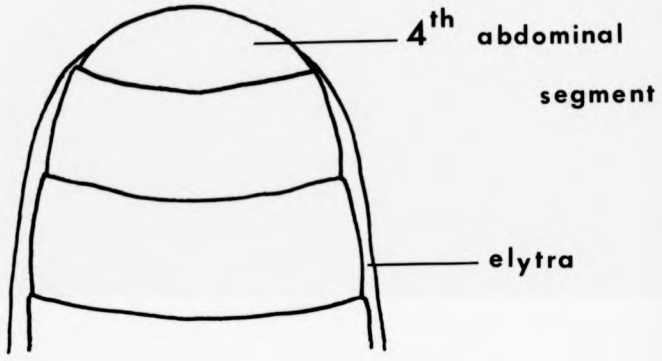
Externally visible sexual characteristics can be used to distinguish between male and female pupae and adults. (Bhattacharya, Ameel & Waldbauer, 1970) Sexing of adult beetles, based as it is upon the comparative shape of the 5th visible abdominal sternite and the width of the intersegmental membranes between the 3rd and 4th sternites (Fig 2.1), is more difficult than pupal sex determination. Pupae, on the other hand, are sexed on the basis of developing genitalia. Both sexes have a small swelling which protrudes from beneath the 7th abdominal sternite. In the male this bears a pair of short, blunt papillae aligned on either side of the mid-line but in the female these papillae are larger and diverge from the latero-caudal angles of the swelling. (Plate 2.1) The majority of insects used for this study were sexed at the pupal stage.

Pupae were collected weekly from a stock colony housed in tanks of bran at 26° C in a light-dark cycle of 14 h light: 10 h dark. The bran diet of larvae and adults was supplemented occasionally with apple. Once separated into sexes, pupae were maintained at 26° C, as above, and examined each day between 9 and 10 am. Beetles were isolated on emergence, that day being taken as day 1 of their adult life.

Fig. 2.1

Ventral abdominal surface of T. molitor

Female



Male

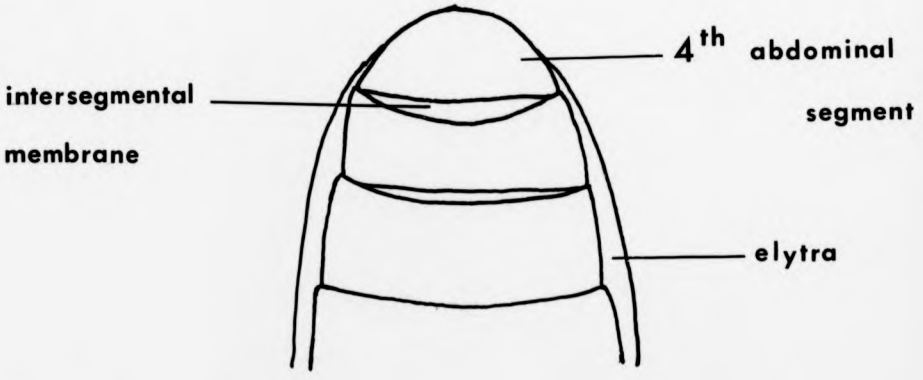


Plate 2.1

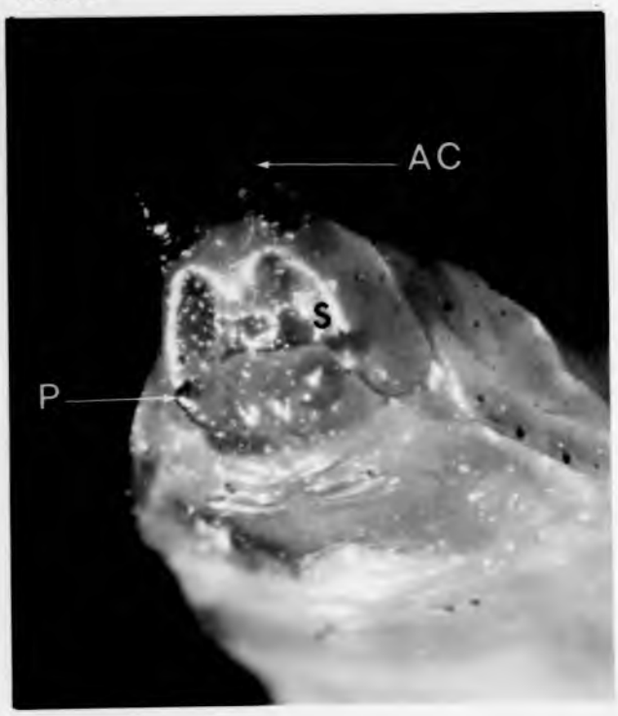
AC - anal cerci

P - papilla

S - 7th abdominal sternite

Plate 2.1 Ventral abdominal surface of T. molitor pupae

female



male

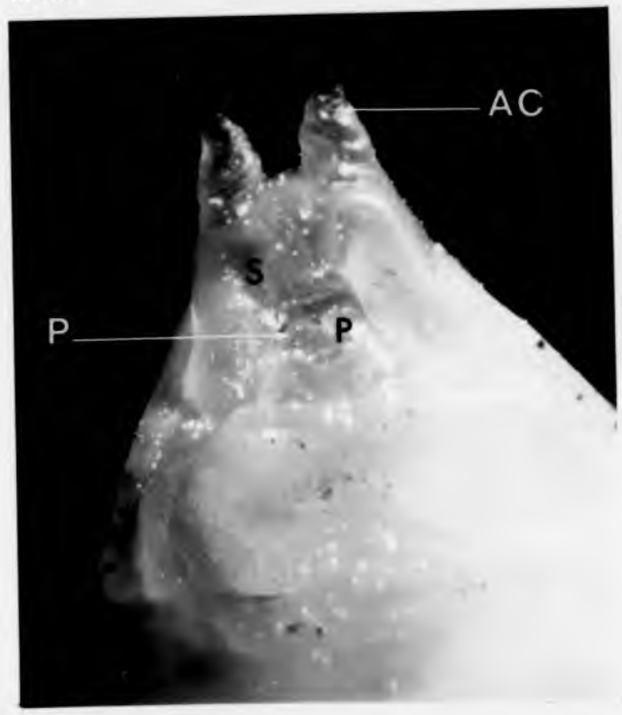


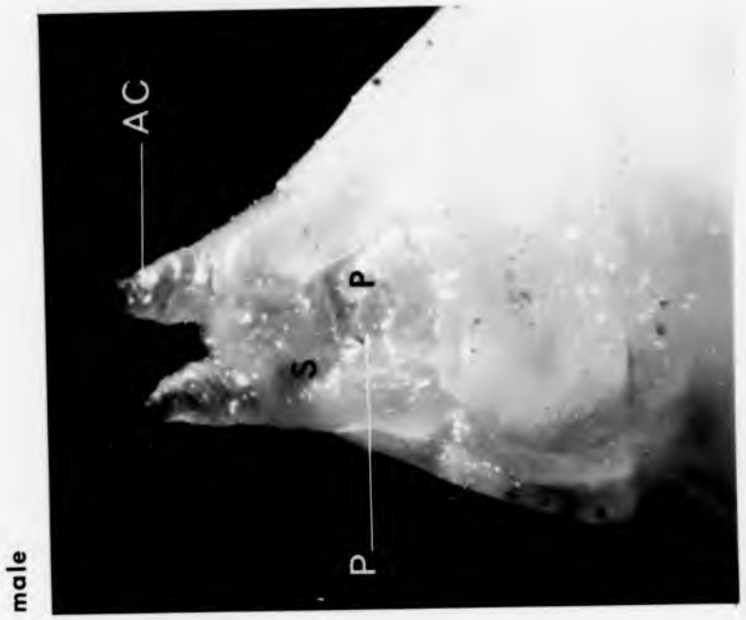
Plate 2.1

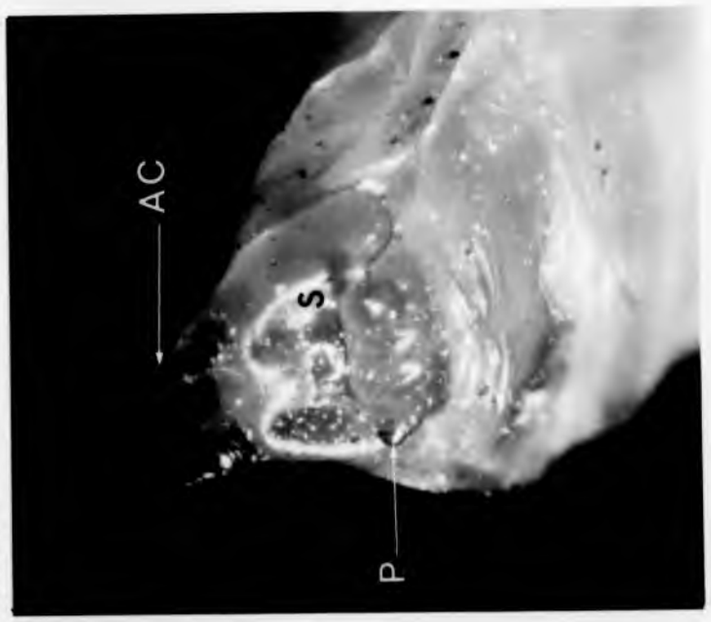
AC - anal cerci

P - papilla

S - 7th abdominal sternite

Plate 2.1 Ventral abdominal surface of *L molitor* pupae





Chapter 3.

HAEMOLYMPH: COLLECTION AND VOLUME DETERMINATION

INTRODUCTION

Collection of insect haemolymph is a time-consuming process, yielding only a fraction of the total volume. The usual methods (such as that described below) involve some form of wounding, the blood being taken up as it is extruded from the wound. Both during this process, and the subsequent handling of the fluid, coagulation and, or, melanization of the haemolymph can occur. Rapid processing for biochemical studies or freezing prior to storage is thus essential.

Coagulation of insect haemolymph can occur very rapidly. In most cases the process is initiated by changes taking place in a class of hyaline haemocytes, the coagulocytes, which are highly sensitive to contact with "foreign" surfaces. Other cell types such as oenocytes and granulocytes are also purported to be involved in haemolymph coagulation in some insects (Crossley, 1979).

In a comprehensive review Gregorie (1974), using phase-contrast microscopy, reported advances made in the determination of cytological features of coagulocytes. He identified 4 different coagulation patterns: (i) cells that extruded small amounts of cytoplasm with concomitant coagulation of plasma as a granular precipitate, (ii) coagulocytes that produced long pseudopodial extensions within the meshwork of which agglutination occurred, (iii) a combination of type (i) and (ii) above and (iv) coagulation with no apparent involvement of haemocytes and dependent upon a humoral reaction. Gregorie (1974) recorded the coagulation sequence in T. molitor as being characteristic of pattern (iii). Crossley (1979)

stated that the centrifugation of haemolymph as soon as possible after its removal, and under conditions that precipitate intact haemocytes, will prevent its coagulation. This has been found to be the case for T. molitor (see below).

Insect blood plasma contains phenolic substances such as tyrosine and dihydroxyphenylamine (DOPA); generalized melanization in vivo does not, however, occur and Crossley (1979) suggested two explanations for this. First, that a proenzyme may be present in an inactive form, and secondly that this enzyme may be separated from its haemolymph substrates by cellular membranes. His review presented evidence for the presence of phenoloxidase and its precursor within haemocytes and discussed its role in cuticle tanning, wound healing and capsule formation. Activation of a phenol-oxidising system in T. molitor has also been described (Heyneman, 1965). During the collection of blood the process of melanization is initiated within the haemolymph.

The measurement of haemolymph volume has been undertaken to investigate three questions which have a bearing on work described in later chapters. First, whether there is a decrease in haemolymph volume with age, as this would result in an apparent increase in blood metabolites although their actual concentrations remain unchanged, secondly, whether haemolymph volume differs between the sexes and thirdly, whether infection with H. diminuta metacestodes alters the blood volume.

MATERIALS AND METHODS

1. Haemolymph collection

Haemolymph samples were collected in the following manner throughout this study. Beetles were held ventral-side uppermost with a blunt pair of forceps whilst the left thoracic leg was severed at the coxa. Drops of extruding haemolymph were collected in a Drummond 10 µl micro-pipette, sealed with sealing wax and immediately centrifuged in a Hawksley micro-haematocrit centrifuge at 12000 g for 5 min. Samples for later use were stored at -20° C for periods of up to 1 month. A few crystals of phenylthiourea (PTU) were placed in the capillary tube prior to sample collection if the haemolymph was likely to be exposed to air for any length of time, as, for example, during electrophoresis or isoelectric focussing.

Prior to analysis the capillary tube sealant, together with the adherent plug of haemocytes, was removed and the amount of haemolymph in the tube measured, (1 mm being equivalent to 0.25 µl).

2. Determination of blood volume

Blood volume was calculated by monitoring the dilution of 1 µl of a solution of ^{3}H-inulin injected and sampled as follows.

(i) Injection technique

An injection system was developed that would accurately deliver 1 µl of fluid with a minimum of damage to the insect. Microelectrodes, made from Pyrex glass tubing which was washed with chromic acid before use, were pulled on a Palmer electrode puller. The electrode tips were "bumped" to an external diameter of 40-50 µm thus giving a tip with sufficient strength to puncture the

intersegmental membrane, yet fine enough to prevent haemolymph leakage from the wound. A 1 cm length of polyethylene tubing was bonded to the end of each microelectrode with Loctite glass-bond. A 10 µl Hamilton syringe was inserted into the polyethylene tube via a nylon chuck, turned to fit the tube exactly and pierced by a hole to take the syringe needle; a small brass clamp tightened round this joint ensured an air tight seal. Both the microelectrode and tubing were filled with injection fluid before assembly. The Hamilton syringe was supported in a perspex holder and the microelectrode held by a manipulator (Plate 3.1). Beetles were secured ventral side uppermost on plasticine and the head bent back slightly to expose the large intersegmental membrane between the 1st and 2nd thoracic segments. Using a binocular microscope, the microelectrode tip was aligned and inserted through this membrane and 1 µl of fluid injected, the tip being held in place for 30 sec before withdrawal. After injection, beetles were returned to Petri dishes with a supply of bran and maintained at room temperature.

(ii)Injection fluid

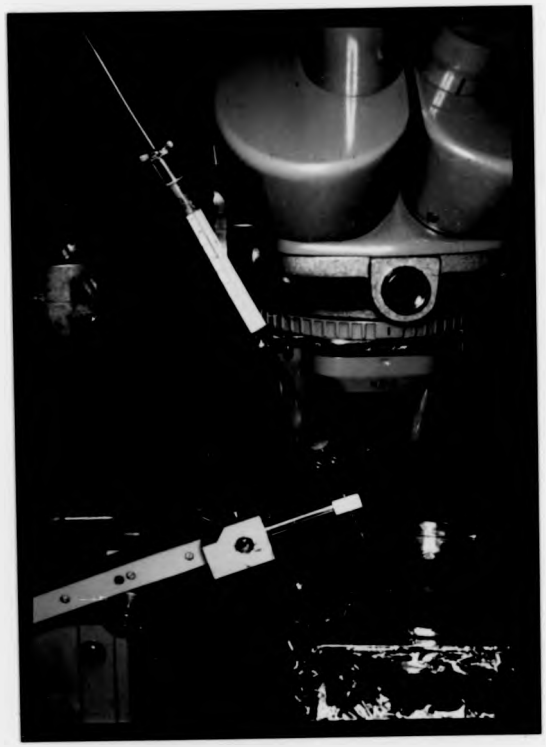
The injection fluid used for blood volume determination was prepared as follows. Tritiated inulin (50 µCi/ml) with a specific activity of 1.84 Ci/mmol (Amersham), was diluted ten times with "Tenebrio saline" A (see Ch. 7) composed of 141 mM NaCl and 42.5 mM KCl. One microlitre of this solution (sp.conc. 0.005 µCi/µl) was injected into individual beetles as described above.

(iii) Blood volume determination

To ascertain the time required for distribution of the labelled inulin throughout the insect, beetles were left for 2, 10, 30, 60 or 120 min before sampling. Two-three µl of haemolymph ~~was~~ were collected from each beetle and the volume noted. The sample was immediately added to 10 ml of scintillation fluid (Luma Gel, Lumac Systems Inc.

Plate 3.1

Injection system



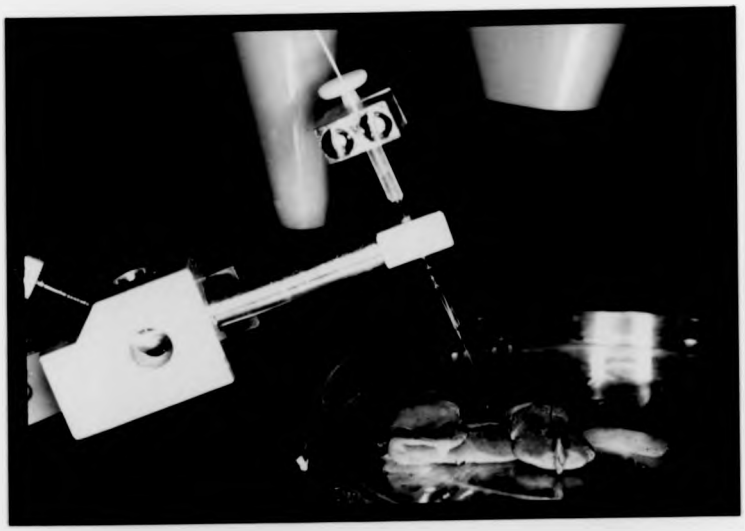
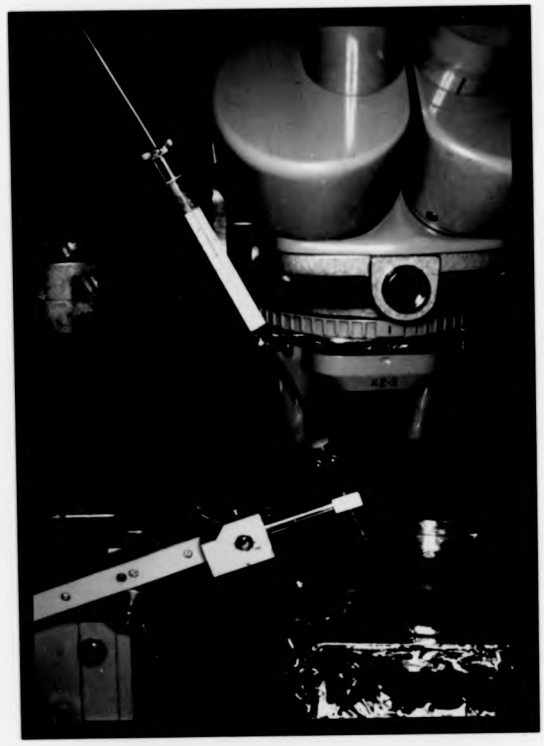


Plate 3.1

Injection system



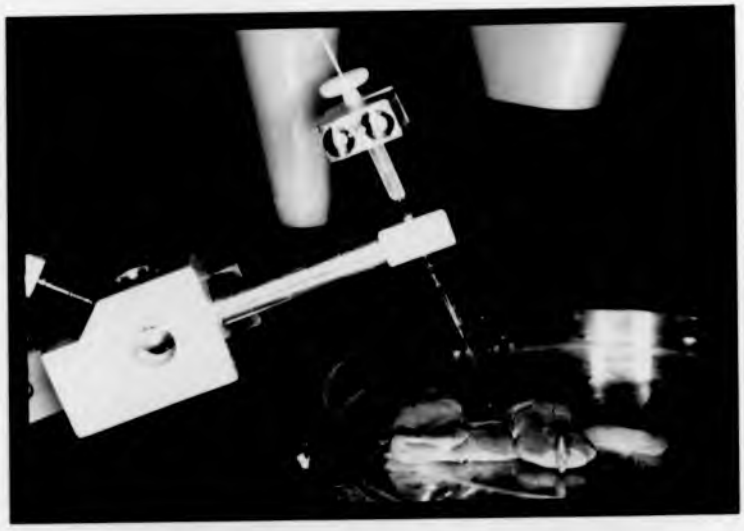
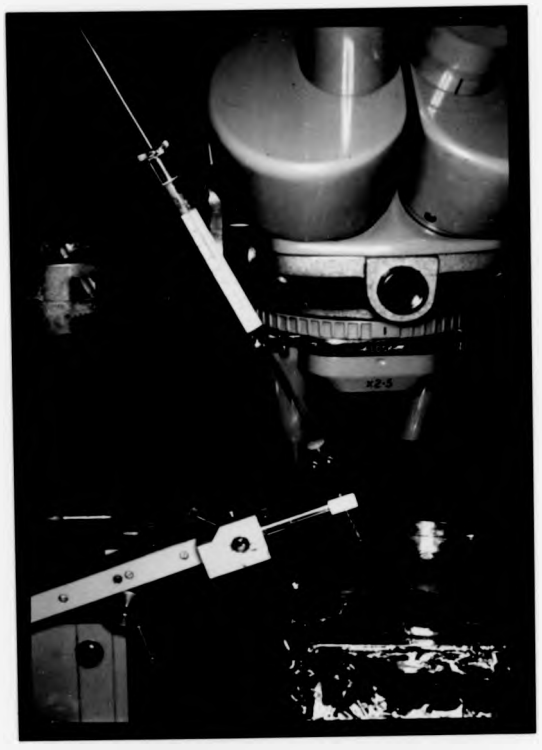
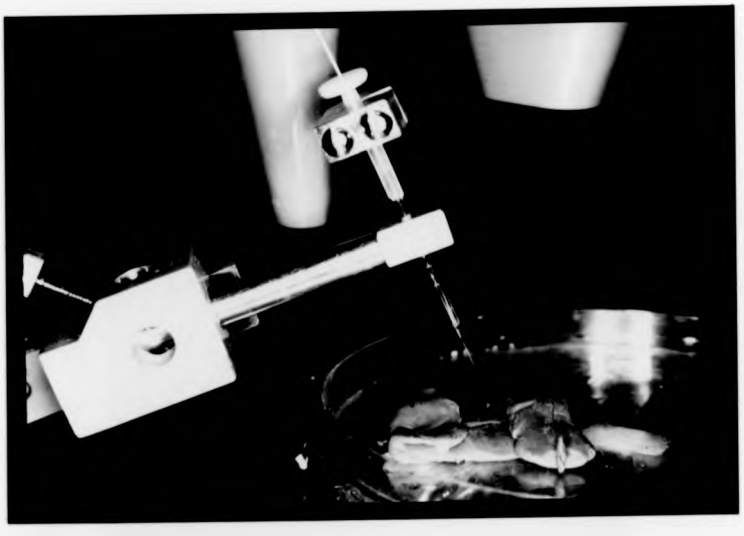


Plate 3.1

Injection system





A. D. Schaesburg, The Netherlands) and the number of counts per minute (cpm) determined using a Packard 2425 Tri-carb liquid scintillation spectrometer set at 1 cycle and counted for 10 min. Male and female non-infected beetles 9, 12, 15 and 20 days-old were investigated and a comparison of haemolymph volume of infected and non-infected 15 day-old insects made. Counts per minute per 1 µl of haemolymph were compared with cpm from 1 µl of injection fluid. After background counts were subtracted from all figures calculations of the dilution factor enabled blood volume to be determined.

(iv) Analysis of the degree of 3H-inulin degradation

Thin-layer chromatography (TLC) was used to check the purity of the 3H-inulin injection solution used. This was felt necessary because any appreciable degradation to sugars which could be readily taken up by the tissues of T. molitor could give rise to an inaccurate blood volume determination. Pre-prepared plates of silica gel (Anchem 250 µm thin Uniplate) were used as an absorbent. Aqueous solutions (2% w/v) of Analar glucose, fructose and inulin, together with the 3H-inulin solutions, were spotted onto the plate. A solvent solution composed of benzene:acetic acid:methanol (20:20:60 v/v) was used to separate the sugars and they were visualized using DAP (5 parts 4% w/v diphenylamine in acetone, 5 parts 4% w/v alanine in acetone and 1 part phosphoric acid). After spraying, the plate was left for 10 min at 105-110° C. The TLC plate was scored into 1 cm squares and each square along the tracks containing 3H-inulin was scraped into 10 ml of scintillation fluid and analysed for radioactivity. One centimeter squares from the edge of the plate were used to determine background counts. Any radioactivity detected in an area other than that predicted by visualization of unlabelled inulin would indicate decomposition of the stock injection solution.

(v) Uptake of ^{3}H-inulin by the tissues

In order to make certain that no inulin had been taken up by the tissues during the incubation period, ovaries, gut and fat body from 5 beetles were removed immediately after haemolymph collection. The gut was slit open and the contents washed out with 0.9% saline. Each pair of ovaries, the gut and fat body were washed 3 times in 50 ml 0.9% saline, homogenised in 0.1 ml 0.9% saline and the resultant homogenates added to 10 ml of scintillation fluid. The radioactivity in the tissues was determined as for haemolymph.

RESULTS AND DISCUSSION

1. Haemolymph collection

Matthews & Downer (1973), investigating the effect of various forms of anaesthetic on blood-sugar levels in the American cockroach, found that hyperglycaemia was induced in all cases but CO_2 treatment, which lowered blood-sugar levels. Handling alone was also found to produce hyperglycaemia and they suggested that the release of a stress-induced hyperglycaemic factor may be involved. In view of this, and other possible effects that anaesthesia may have on haemolymph biochemistry, it was decided for the purpose of this study to collect haemolymph both without the use of anaesthesia and with the minimum of handling of the animals. Samples were therefore routinely obtained within 1 min of removing the beetle from its Petri dish. A time factor was also found to be important with respect to melanization and coagulation of haemolymph. In the majority of studies reported here samples were centrifuged, frozen and stored within 8 min of collection. No coagulation or melanization was observed within that period; however, a dark brown ring formed at the

top of the sample after exposure to air at room-temperature for 20 min, and a general darkening of the haemolymph was also observed. Addition of a crystal of phenylthiourea (PTU) to samples to be kept at room-temperature for more than 20 min was found to inhibit this melanization. Hayes, Johnson & Schechter (1975) reported the inhibition of browning by several agents such as mercaptoethanol, thioglycolic acid and cysteine, which protect sulphydryl groups, and also by some reducing agents for example sodium hyposulphite and ascorbic acid. They concluded that this was consistent with the hypothesis that oxidation of sulphydryl- to disulphide- groups is required for the oxidation of the melanization enzyme system. Phenylthiourea was found to be the most effective inhibitor of melanization investigated in their study.

Sternburg & Corrigan (1959) described a method for the rapid collection of cockroach blood by gentle centrifugation of insects following clipping of legs and antennae. This was adapted in this study for use with the much smaller T. molitor, but eventually abandoned in favour of the more reliable blood collection method described above. The majority of beetles yielded 2-3 µl of haemolymph although larger samples of 6-8 µl were occasionally collected. These volumes represent only a small proportion of the total haemolymph, fifteen day-old females, for example, contained a mean blood volume of 26.3 µl. Other authors have also reported the collection of approximately one tenth of the total blood volume available. Thus Khan, Koopmanschap, Privee & De Kort (1982) collected 5-10 µl blood per Colorado potato beetle, yet calculated the mean haemolymph volume to be 50.9 µl.

2. Determination of 3H-inulin degradation

Glucose was visualized on the TLC plate as a blue spot which had an Rf value of 0.86, fructose as an orange spot, Rf 0.79 , and inulin as a red spot tailing for 6 mm from the origin. Examination of the track containing radiolabelled inulin revealed two peaks of activity, the major one was found in the area 10-20 mm from the origin and the minor one, representing 12.2% of the total counts, 60-70 mm from the origin giving an Rf value of approximately 0.92. Inulin is a storage polysaccharide which consists of D-fructose residues with β 2-1 linkages. The occurrence of radioactivity in the same position on the plate as fructose would suggest that some degradation of the injection solution has occurred. It was not, however, felt that the proportion of sugar available for uptake by the tissues as a monosaccharide was sufficient to warrant any adjustment of the dilution calculations.

3. Tissue uptake of inulin

The sequestration of inulin from the blood by developing oocytes of the milkweed bug has been measured and used as an index of vitellogenesis by Aldrich, Soderlund, Bowers & Feldlaufer (1981). They did not record the shortest incubation time used, but describe a sharp increase in dpm/ovaries 2 days post-injection. An examination of ovaries, guts and fat bodies from 5 beetles exposed to 3H-inulin during a 10 min incubation period showed radioactivity to be at background levels in all cases. It was thus concluded that no removal of inulin or its degradation products had occurred during the time period used to determine blood volumes in this study.

4. Blood volume determination

Haemolymph volumes, calculated for different times post-injection, are shown in Table 3.1. Collection of haemolymph 2 min post-injection proved impossible. The wound made by the microelectrode tip had not healed by this time and thus application of gentle pressure during blood collection caused leakage from the wound rather than the severed leg. After a period of 10 min wound healing had progressed sufficiently to prevent haemolymph leakage at this point.

Mean blood volumes calculated from samples taken 10 and 60 min after injection did not differ significantly. Samples taken 30 and 120 min post-injection however, yielded higher cpm and thus calculations of ^{3}H-inulin dilution gave mean blood volumes significantly lower than those of beetles sampled 10 or 60 min post-injection (Table 3.1).

Beetles used for these determinations were selected at random from the stock colony and thus differences in age, sex and size of insect could account for variations in blood volumes recorded. It was decided to use 10 min post-injection as the sampling time for blood volume calculation. Only beetles of known age and sex were used, and those outside the size range 13-15 mm were rejected.

Mean haemolymph volumes calculated for non-infected beetles 9, 12, 15 and 20 days-old and infected 15 day-old beetles are recorded in Table 3.2. No significant differences were detected between the sexes at any age examined, nor between infected and non-infected 15 day-old beetles. A decline in blood volume with age was however noted after 12 days, both female and male 20 day-old insects contained significantly less haemolymph than 15 day-old beetles. This finding is consistent with the observation that haemolymph is

Table 3.1 Blood volumes of non-infected beetles, sampled at various times post injection.

Time	n	Volume in µl ± S.E.
2 min	10	-
10 min	10	32.74 ± 3.9
30 min	10	† 14.10 ± 1.1
60 min	9	22.50 ± 7.9
120 min	10	† 16.21 ± 2.2

† Values significantly different from those for 10 min $p(<0.001)$

Table 3.2 Blood volumes of non-infected and infected T. molitor in µl ± S.E.

	Non-infected								Infected	
	n	9 day	n	12 day	n	15 day	n	20 day	n	15 day
♀	10	29.9 ± 0.3	13	31.2 ± 1.9	16	26.3 ± 1.9	9	*18.6 ± 2.7	13	23.9 ± 1.8
♂	8	27.7 ± 0.4	11	31.3 ± 2.5	7	30.0 ± 2.9	11	*19.9 ± 2.3	14	25.8 ± 2.8

* Values significantly different from other values for the same sex ($p<0.05$)

more difficult to extract from 20 and 30 day-old beetles, and must be considered when comparisons of blood metabolites at various ages are examined. In this study metabolites such as haemolymph soluble proteins (Ch. 4) and haemolymph free amino acids (Ch. 10) have been expressed as units per µl blood. As blood volume declines with age, soluble components would thus appear to increase in concentration.

With the exception of mated beetles used for fecundity studies (Ch. 9), adult T. molitor examined in this work were maintained solely on bran (see Ch. 2). The cryptonephridial complex of the mealworm beetle, which was described by Ramsey (1964), Grimstone, Mullinger & Ramsey (1968) and at the ultrastructural level, by Meyran (1982), removes water from the faeces, thus allowing the insect to exist on a dry diet. The life-span of beetles maintained on a dry diet was, however, considerably shorter than that of those maintained on a diet supplemented with apple (the majority of insects did not survive for 30 days). It is thus considered likely that the water absorbtive ability of the cryptonephridial complex is unable to compensate completely for water loss experienced by adult beetles maintained on a dry diet, thus leading to the significant reduction in blood volume observed in this study in 20 day-old beetles.

Chapter 4

TOTAL HAEMOLYMPH SOLUBLE PROTEINS IN TENEBRIO MOLITOR

INTRODUCTION

Some of the many reports of the effects of parasites upon total haemolymph soluble proteins (THSP) have been discussed in Ch. 1, where it was noted that a depletion of haemolymph protein is commonly associated with infection. Some host haemolymph constituents are undoubtedly utilized by metacestodes of H. diminuta to meet their nutritional requirements; it is however a matter of conjecture whether soluble proteins are utilized by the parasite.

The tegument of adult cestodes is not readily permeable to macromolecules such as proteins and Lumsden, Threadgold, Oaks and Arme (1970) were unable to confirm the reported uptake of colloidal particles by H. diminuta (Rothman, 1967). Recent investigations by Hopkins & Law, (1978) and Threadgold & Hopkins (1981) have, however, demonstrated the occurrance of endocytosis in pleurocercoids of Schistocephalus solidus and Ligula intestinalis and also in adult S. solidus. There is considerable evidence, summarised by Pappas (1983), that cyclophyllidian metacestodes are permeable to high molecular-weight solutes, host proteins having been identified within cysticerci and hydatid cysts, and endocytosis observed in cysticercus of Taenia crassiceps (Threadgold & Dunn, 1983). The uptake of protein or other macromolecules by metacestodes of H. diminuta has not been demonstrated and, in preliminary experiments, Arme & Richards (unpublished data) were unable to show uptake of either free or liposomally entrapped ruthenium red. This metacestode can, however, absorb a variety of low molecular-weight organic nutrients by specific membrane transport mechanisms (Arme & Coates, 1973; Arme,

Middleton & Scott, 1973; Jeffs & Arme, 1983 & 1985b; Phillips & Arme, 1983) and protein synthesis occurs following amino acid absorbtion (Jeffs & Arme, 1984).

The effects of the parasite upon THSP of male and female beetles at various times post-infection has been investigated and the results of these studies are reported below.

MATERIALS AND METHODS

1. Sample collection

Beetles were cultured, sexed and infected as described previously. Samples of haemolymph were collected (see Ch. 3) from virgin infected and non-infected beetles 9, 12, 15, 20 and 30 days post-emergence and from mated females 15, 20 and 30 days post-emergence, centrifuged and immediately stored at -20° C. Following blood sampling, beetles were dissected in 0.9% (w/v) sodium chloride the sex of the beetle confirmed and the spermatheca of paired females examined for the presence of sperm, unfertilized beetles being excluded from the study of mated females. The number of metacestodes harboured by infected beetles was recorded.

2. Protein determination

For the quantitative determination of protein, a known volume of cell-free haemolymph (2-3 µl) was added to 3 ml of ice-cold 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 2000 g for 15 min. The precipitate was washed in a further 3 ml of ice-cold TCA and, after centrifugation as above, was dissolved in 0.5 M sodium hydroxide at a ratio of 1.6 ml NaOH / protein from 1 µl of haemolymph. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) as follows: Four ml of Lowry's reagent (1 part 1% w/v copper

sulphate, 1 part 2.68% w/v potassium sodium tartrate and 100 parts 2% w/v sodium carbonate) were added to a 1.6 ml aliquot of the protein solution and the mixture left at room temperature for 10 min. One volume of Folin phenol reagent (BDH) was diluted with two volumes of distilled water and 0.4 ml of this solution was quickly added to each assay tube, immediatly mixed with a vortex mixer and maintained at room temperature for 30 min. A set of standard tubes was prepared containing bovine serum albumin (BSA) dissolved in 1.6 ml of 0.5 M sodium hydroxide to give concentrations of, 10µg, 20µg, 50µg, 100µg, 150µg and 200µg BSA. A blank tube containing 1.6 ml of 0.5 M NaOH alone was also prepared and the standards and blank were assayed with the samples. Absorbance was read against the blank at 750 nm using a Cecil CE Grating Spectrophotometer. Optical densities of the standard solution were plotted and protein values of the sample tubes calculated from this standard curve.

To determine the suitability of the above procedure for protein determination a recovery control was performed using, instead of haemolymph, 1 µl samples from aqueous solutions of BSA (30 mg/ml). Protein recovery from this sample was a mean of 89% of the amount originally present.

3. A comparison of cell-free and whole haemolymph

A comparison of THSP from cell-free and whole haemolymph was made in order to ascertain the protein content of the haemocyte plug removed from samples before analysis. Two blood samples were collected from each beetle, one was treated as described above and the other sealed and stored without centrifugation. The protein content of both samples was determined by the Lowry technique and the results compared.

4. A heat-killed egg control

As an additional control heat-killed eggs of H. diminuta were fed to 20 day-old female beetles using the method described in Ch. 2, and a comparison made of THSP from females exposed to live eggs, dead eggs and apple pulp alone, using the Lowry method of protein determination outlined above. The parasite eggs were killed by heating in a water bath at 60° C for 10 min and their viability checked in the following way: a sample of heat-treated eggs was removed and the egg suspension placed on a microscope slide with one drop of amylase (200 mg/ 50 ml KRT (see Appendix 8)) and one drop of trypsin (200 mg/ 50 ml KRT). A cover slip was applied and tapped to rupture the egg shells (for a discussion of egg hatching see Ch. 2). Untreated and heat-killed eggs were observed at 100x magnification for 1 h. Onchosphere hook movement was observed within 1 min in normal eggs, escape from the inner envelope being completed in 6 min. The shells from the heat-killed eggs were ruptured by the cover-slip and the gelatinous layer was seen to swell. After 30 min this layer had disappeared, however no hook movement was observed after 1 h.

5. A fluorimetric determination of protein

A fluorimetric determination of haemolymph protein was undertaken on haemolymph from 20 day-old beetles (a modification of the method of Bollen, Stein, Dairman & Udenfrienf, (1973), developed by R. Lobley (personal communication)). Fluorescamine (4-phenylspiro [furan-2(3H), 1'- phthalan] 3,3'-dione, trade name "Fluram" Roche) is a non-fluorescent compound which forms intensely fluorescent derivatives with primary amines. Haemolymph samples, collected as above, were diluted 100x with distilled water and 50 µl aliquots mixed with 250 µl bicarbonate buffer (0.15 M $NaHCO_3$ and 1% w/v SDS, pH 10). This mixture was incubated at room temperature for 10 min.

A 50 µl standard (10 mg/ml bovine serum albumin solution (Pentax)) and a 50 µl water blank were set up as above. Whilst holding the test tube in a vortex mixer 100µl fluorescamine (0.3 mg/ml in acetone) was added by means of a Repette. Solutions were incubated at room temperature for 10 min, then 0.6 ml distilled water was added and mixed well. Fluorescence was read at λ_{ex} = 395 nm, λ_{em} = 490 nm and the amount of protein in the sample given by:

$$\text{Protein} = \frac{F_x \times S}{F_s} \quad \mu g/ml$$

where:

F_x is the fluorescence given by the sample blank

F_s is the fluorescence given by the standard - blank

S is the concentration of the standard in µg/ml.

RESULTS AND DISCUSSION

Data concerning Lowry protein determinations in virgin beetles are summarised in Table 4.1, mean values varying with age from 62.0-81.3 mg/ml in non-infected females and 36.1 - 54.3 mg/ml in non-infected males. In their review of haemolymph composition, Florkin & Jeuniaux (1974) observed that protein concentration of insect blood is generally higher than that of other invertebrate body fluids and quote 30-40 mg/ml as the average protein content of haemolymph from coleopterans. The majority of THSP concentrations determined in this study fall outside the range quoted by Florkin & Jeuniaux (1974) and indeed are higher than all of the protein N values tabulated by Dittmer (1981) in his survey of insect haemolymph constituents. Differences in both the technique used to measure protein concentration and the different developmental stages

Table 4.1 Total haemolymph protein concentrations of infected and non-infected adult Tenebrio molitor.

	Total haemolymph protein (mg/ml) ± S.E.				
Beetle age in days post emergence	9	12	15	20	30
Sample size	10	18	20	20	17
Non-infected females	62.0±8.2	81.3±5.3	64.2±6.6	69.8±6.2	77.7±5.6
Infected females	65.2±6.7	88.1±6.8	94.2±7.2	99.3±8.5	105.6±9.5
Protein elevation in infected beetles	5.2%	8.4%	46.7%*	42.3%*	36%†
Sample size	10	10	14	16	10
Non-infected males	36.1±2.9	54.1±9.5	54.3±5.4	37.7±3.5	48.2±8.8
Infected males	31.4±2.7	51.3±4.5	54.2±5.6	38.9±3.8	42.2±3.8

* Significant difference P = 0.01

† Significant difference P = 0.02

investigated could account for the wide variation quoted in the literature for THSP concentrations in what is *per se* a very diverse class.

In their description of THSP determination many workers do not report that they separate haemocytes from plasma, yet the cellular fraction of haemolymph contains abundant protein (Crossley, 1979). Whole haemolymph from *T. molitor* of unspecified age and sex was found, in this investigation, to have an average of 28 mg/ml more protein than cell-free blood. Due to the probability that freezing and thawing disrupts haemocytes and leads to leakage of cellular proteins, all blood samples used for analysis were centrifuged before storage. It was not ascertained whether any haemolymph coagulation occurredduring centrifugation and thus the haemocyte plug, removed after storage and prior to analysis, may have contained haemolymph proteins involved in a coagulation reaction and originally component parts of the total soluble fraction. The concentration of any such proteins has been disregarded for the purpose of this study.

Mordue (1965c), using insects reared under crowded conditions, determined haemolymph protein concentration in 14 day old virgin *T. molitor* to be 11.3 + 0.09 mg/ml. The discrepancy between his results and those obtained in this work could be due to the differences in rearing conditions or be the result of the different techniques used for protein determination. Mordue (1965c) measured haemolymph protein concentration by the Biuret method, using a calibration curve constructed from measurments of diluted haemolymph, a micro Kjeldahl method having been employed to calculate the protein nitrogen content.

The Biuret test for the quantitative determination of proteins is based upon the reaction between compounds containing two or more peptide bonds and dilute copper sulphate in alkaline solution. The

characteristic purple colour is produced by the copper complex formed with four nitrogen atoms, two from each of two peptide chains. This test is consistant for different proteins but requires high concentrations of protein (1-20 mg). The Folin-Ciocalteu test (Lowry, Rosebrough, Farr & Randall, 1951) used in this study is more sensitive than the Biuret test (5-10 µg can be analysed). The protein is assayed by two colour-forming reactions, the first occurs when the protein forms a complex with alkaline copper sulphate and the second is the result of the reduction of phosphomolybdate-phosphotungstate salts in the reagent by the tyrosine and tryptophan present in proteins. If the proportion of these amino acids present in a particular protein differs from the proportion present in the protein chosen as a standard, the difference in colour intensity produced by the same concentration of protein will lead to inaccuracies in the determination.

Subhashini & Ravindranath (1980) made a comparison of protein values in crab haemolymph determined by three different methods. Using BSA as a standard in each case, the micro Kjeldahl method (Peters and Van Slyke, 1932) gave values lower than the Biuret method and Lowry methods. The reason for this was ascribed to the high lipid and polysaccharide content of the haemolymph proteins which would depress the %N content and render the conversion factor inaccurate. Subahashini & Ravindranath (1980) found that the Lowry and Biuret methods gave similar results, however, Levenbook & Bauer (1980) found that the use of BSA as a standard for the Lowry technique led to an over estimate of plasma protein concentration in the blowfly, Calliphora stygia, when compared with calliphorin (the main storage protein of C. stygia) used as a standard. Calliphorin has an exceptionally high tyrosine content, as have many insect proteins (Sroka & Bradleigh, 1978), and the use of BSA as a reference

protein in the Lowry procedure could therefore lead to an exaggeration of insect haemolymph protein concentration. Although an analysis of the amino acids of T. molitor haemolymph proteins has not been performed De Loof & De Wilde, (1970) examined the vitellogenic protein of another coleopteran, Leptinotarsa decemlineata, and found tyrosine to represent only 3.76 molecular %. However, as the work was based on acid hydrolysis low values for tyrosine would be expected.

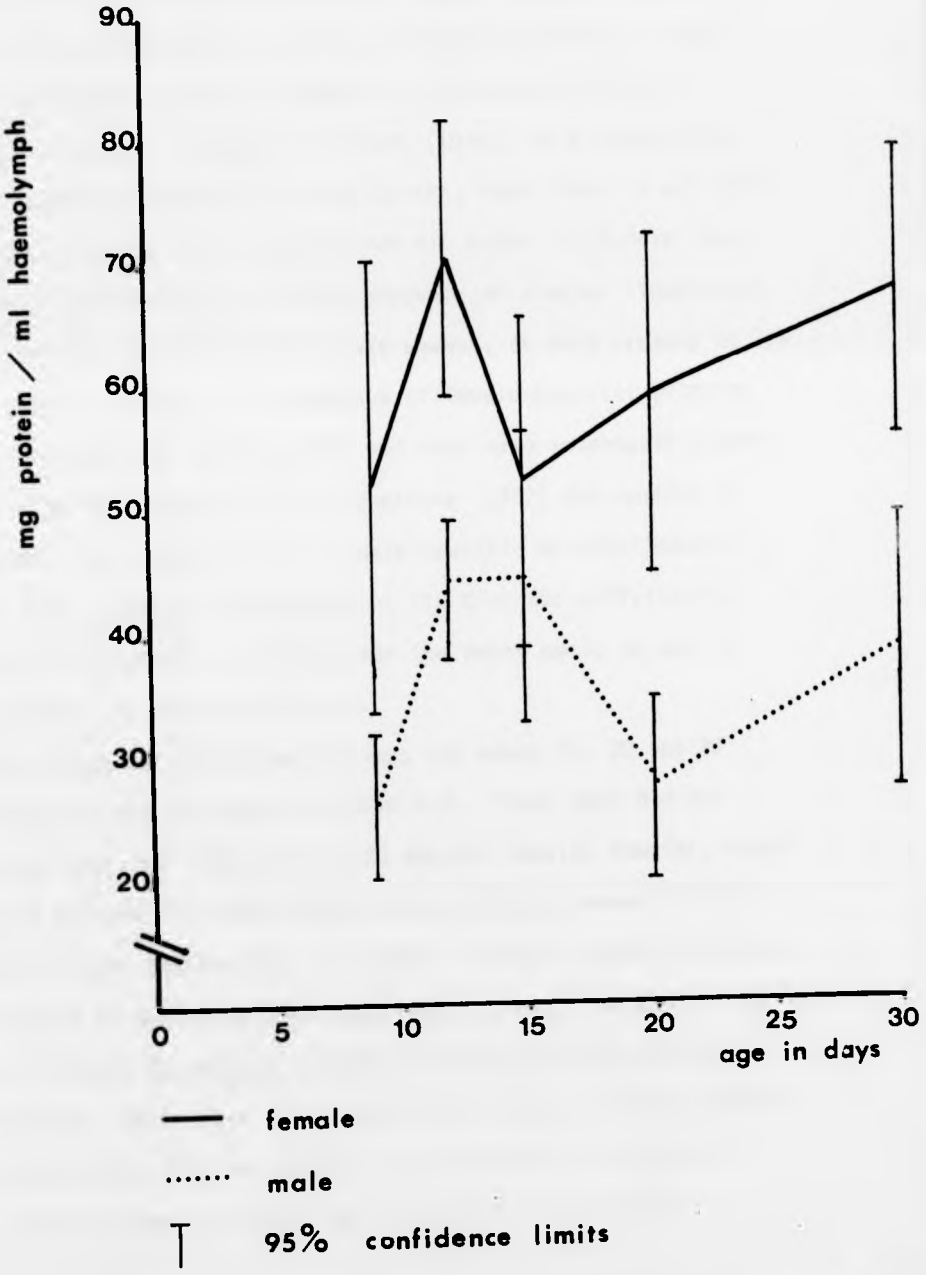
A fluorimetric determination of THSP was thus undertaken on a limited number of samples to compare measurments obtained using these two different techniques. The results fell within the range found using the Lowry method. It was therefore decided to standardise upon the Lowry technique, using BSA as a standard for this analysis.

The variation in THSP with age in non-infected male and female beetles is represented in Fig. 4.1. In male beetles a peak was detected at 15 days, concentrations then dropped significantly ($p < 0.02$) at 20 days and rose to 48.2 mg/ml in 30 day-old beetles. A similar pattern was observed in female beetles, a peak of 81.3 mg/ml occurred earlier however, and a significant decrease ($p < 0.05$) had taken place by day 15. Many authors have reported variations in THSP during the development of holometabolous insects (for example, Kinnear & Thompson 1975; Brown, 1980 and Arbuthnot, Cantrill & Hepburn, 1983) A peak is generally reached during the last larval instar, protein concentration then declining with adult age (Wyatt & Pan, 1978). No fall in protein concentration with age was detected in this study. The significant decrease in blood volume reported in the previous chapter suggested that the older beetles were becoming desiccated and this could give the appearance of a steady or rising protein concentration measured on a w/v basis when, in fact, protein per beetle was constant or declining.

Fig. 4.1

Haemolymph protein concentration of

T. molitor



Male beetles were found to have significantly lower concentrations of haemolymph protein than females at all ages investigated, with the exception of 15 day-old beetles (see Fig. 4.1). In their review of insect plasma proteins Wyatt & Pan (1978) stated that, during vitellogenesis and pre-vitellogenic stages, haemolymph from the majority of female insects contains substantially more protein than that from males and that, in many cases, fluctuations in THSP in females corresponded with the reproductive cycles. Whitmore & Gilbert (1974), in a comparative study of haemolymph proteins in Lepidoptera, found that in all seven species analysed the THSP concentration was higher in females than males. They attributed this to the presence of a major lipoprotein in the haemolymph of females which was absent, or only present in low concentrations in males. The presence of female-specific proteins was first reported by Telfer (1954) and they have subsequently been identified in many species. Both Laverdure (1972) and Harnish & White (1982) have identified two female-specific or vitellogenic proteins in T. molitor and the possibility that the differences, detected in this work, in THSP between the sexes could be due to their presence, is examined in Ch.6.

The results of THSP determinations for mated 15, 20 and 30 day-old insects are expressed in Table 4.2. These data did not differ from that for virgin 15 and 20 day-old females however, mated 30 day-old beetles had significantly lower protein concentrations than their virgin counterparts ($p < 0.001$). Mordue (1964c), using the Biuret method to determine THSP (see above), found that both mated and virgin female T. molitor ,showed a maximum haemolymph protein concentration 8 days after emergence, mated females having a higher concentration than virgins at this age although at 3, 14, and 21 days, concentrations in virgins were greater. Considerable

Table 4.2 Haemolymph protein concentration of mated female infected and non-infected *T. molitor*.

	Total haemolymph protein mg/ml ± S.E.		
Beetle age in days post-emergence	15	20	30
Sample size	10	10	22
non-infected	63.9 ± 8.11	67.9 ± 8.5	40.2 ± 2.8 *
infected	70.4 ± 5.3	89.4 ± 11.9	63.2 ± 5.3

*Sig. difference $p < 0.001$

differences existed between the rearing conditions reported by Mordue and those used in this study. He maintained beetles in crowded conditions (12-16 insects per jar) at 28° C whereas beetles used in this investigation were kept singly or in pairs at 26° C. In addition to the 2° C temperature difference, the initial two-day starvation period may have resulted in a retardation of maturation, thus accounting for the delay in peak protein concentration from 8 days (Mordue, 1964c) to the 12 day peak observed in this study.

Virgin females examined in this investigation laid 0-4 eggs between 6 and 15 days post-emergence whereas mated females maintained as a pair laid 4-7 eggs per day from day 6-30 post-emergence (see Ch. 9). This continuous egg production by mated females would result in a considerable uptake of haemolymph protein by the ovaries and this may account for the significantly lower THSP concentration detected in 30 day-old mated beetles in this study and seen in 14 and 21 day-old females by Mordue (1964c).

Comparison of THSP from infected and non-infected female beetles revealed no significant differences at 9 or 12 days post-emergence. By 15 days however, the haemolymph protein concentration in infected virgin females had increased to a level 46.7% higher than their non-infected counterparts and significant differences between parasitized and non-parasitized females persisted in beetles 20 and 30 days-old. THSP from infected mated females was also significantly elevated in 30 day-old insects. In males no such differences were observed. (Table 4.1 and 4.2).

The results of a comparison of protein determinations on haemolymph from 20 day-old females fed live eggs, heat-killed eggs or apple alone are summarised in Table 4.3. A highly significant ($p=<0.001$) elevation in THSP concentration was again detected in infected beetles and this elevation was, surprisingly, also evident

Table 4.3 Haemolymph protein concentration in 20 day-old female *T. molitor* fed live on heat-killed eggs.

	n	Haemolymph protein mg/ml ± S.E.
live eggs	35	98.1 ± 4.2
heat-killed eggs	35	89.7 ± 3.4
non-infected controls	31	70.1 ± 4.7

live eggs/dead eggs	NS.
dead eggs/non-infected	$p < 0.01$
live eggs/non-infected	$p < 0.001$

in beetles previously exposed to heat-killed eggs ($p=<0.01$). The mean value for protein concentration in females fed heat killed eggs was lower than that for insects fed live eggs. However, this difference is not significant and could be due to the failure of some of the former group to ingest the heat-killed eggs. Examination of faecal pellets for the remains of egg shells or undamaged eggs would have ascertained whether this were indeed the case, but this was not performed.

Data from 15, 20 and 30 day-old infected females were subjected to a correlation analysis which revealed that, within a single beetle age group, there was no relationship between elevation of protein concentration and the number of parasites present. The product-moment correlation coefficient, r, of each analysis is presented in Table 4.4, and a representative scatter graph is shown in Fig. 4.2. The number of parasites harboured ranged from 2-448 metacestodes per beetle.

In marked contrast to the majority of larval cestode/invertebrate host relationships investigated, these findings for females indicate a significant elevation in haemolymph proteins in beetles containing cysticercoids aged 12 days or more. At this age parasites are infective to the final host and almost fully developed, only relatively minor changes, largely involving fibre deposition, taking place in older cysticercoids (see Ch. 3). Thus, these pathophysiological changes in the host are first detected after the time when the metabolic demands of the parasite are presumably greatest.

This excess protein could originate from either host or parasite. It is considered that the latter is unlikely however for the following reasons: first, as stated above, elevated THSP concentrations are not evident during the period when the

Fig. 4.2

Relationship between parasite burden and haemolymph protein in 15 day females

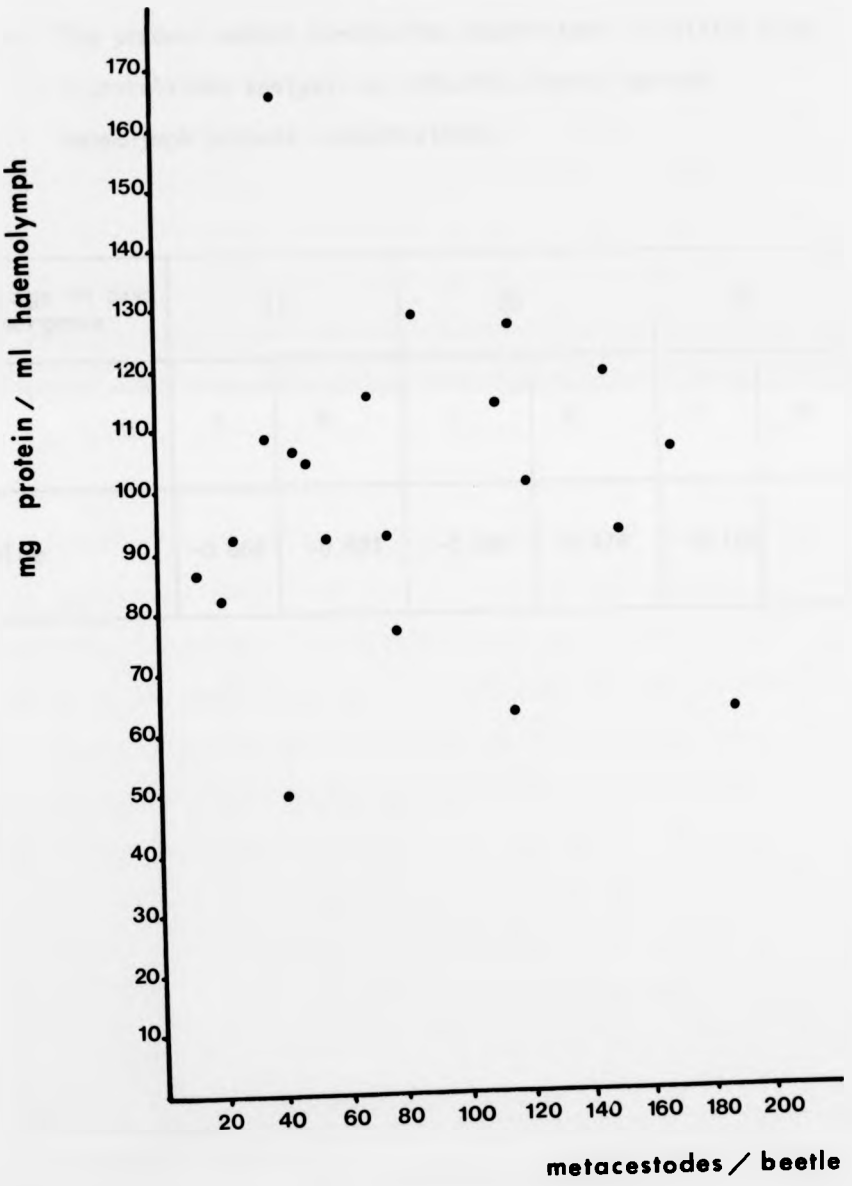


Fig. 4.2

Relationship between parasite burden and haemolymph protein in 15 day females

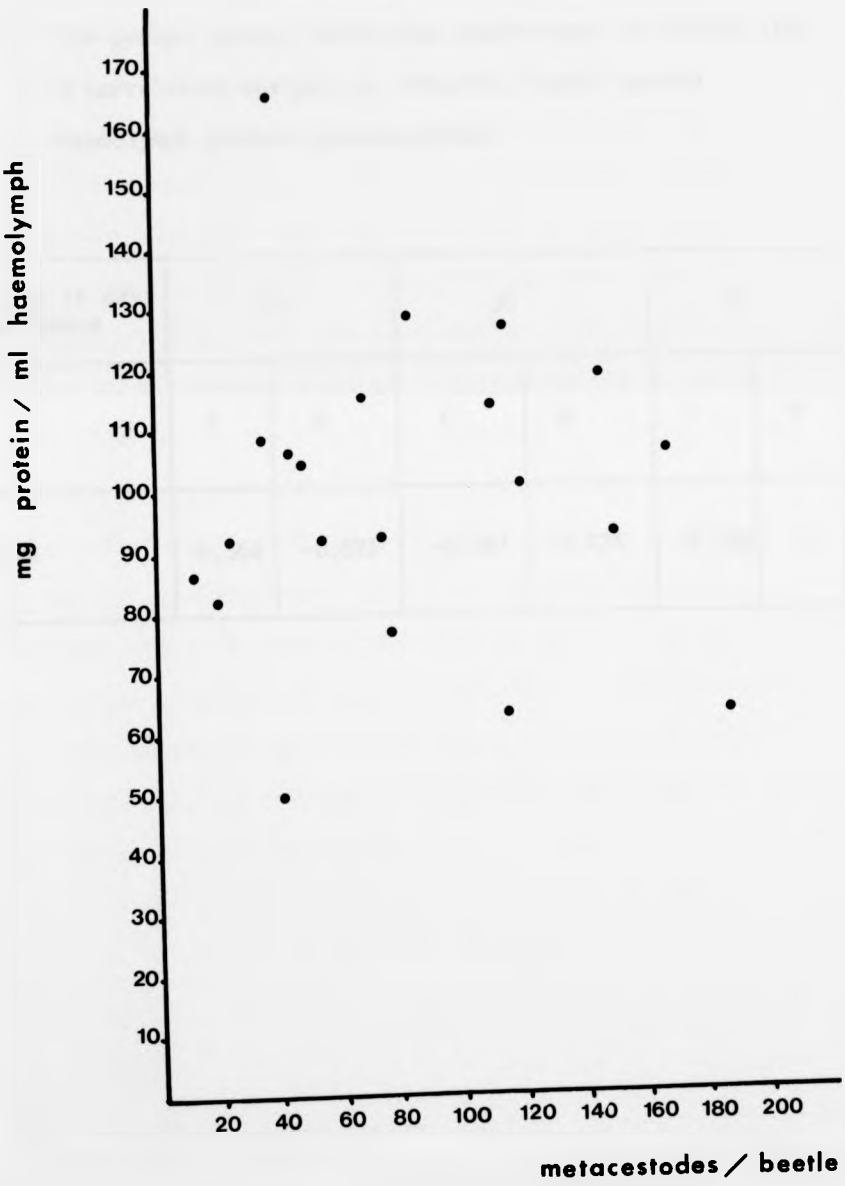


Table 4.4 The product moment correlation coefficient calculated from a correlation analysis of infection levels against haemolymph protein concentrations.

Beetle age in days post-emergence	15		20		30	
Sex	F	M	F	M	F	M
'r' value	-0.068	-0.521	-0.087	-0.474	-0.126	-

metacestodes are metabolicaly most active, secondly, the increase in protein concentration is not proportional to the number of parasites present, thirdly, the alteration in THSP is only detected in female beetles and fourthly, the effect is also evident in females that have ingested heat-killed eggs containing larvae that are unable to penetrate the gut and develop in the haemocoel.

It has been suggested that the host reaction to invading organisms, involving haemocytes and/or a humoral response, may be responsible for changes in the composition of haemolymph protein and examples of this have been discussed in Ch. 1. It is unlikely that the protein elevation, described above, is due to the production of protein associated with a host defense reaction since the haemolymph of both sexes is not affected equally. The fact that only female insects are involved indicates that the effect is associated in some way with inherent physiological differences between the sexes.

One such difference is the presence of vitellogenic proteins in female T. molitor mentioned above. In order to investigate the hypothesis that the protein elevation observed as a consequence of infection of female beetles was due to an increase in female-specific proteins alone, separation and quantification of haemolymph from infected and non-infected insects was undertaken. This work is described in the following chapters.

Chapter 5

SEPARATION OF HAEMOLYMPH PROTEINS: I. ISOELECTRIC FOCUSSING

INTRODUCTION

Considerable difficulties were encountered whilst attempting to separate the haemolymph proteins of *T. molitor*. In view of this the following chapter has not been written in the traditional way, but as an account of the development of the techniques employed. The results achieved at each stage are thus included within the discussion of the methods used.

Proteins have a net charge which can vary according to the pH of their environment and which changes in a continuous manner as proteins pass through a pH gradient (Fig. 5.1). Two electrophoretic techniques were investigated *viz*, isoelectric focussing (IF) and polyacrylamide gel electrophoresis (PAGE). A brief description of the principles underlying these techniques is given below.

1. Electrofocussing

When proteins are placed in an electric field applied across a stable pH gradient they migrate towards the anode or cathode, depending upon their charge. Each protein attains a zero charge at a pH unique to itself (Fig. 5.2), this is its isoelectric point (pI). Any diffusion from this point will be countered, as the protein will then gain a net charge and thus migrate back into its own pI zone. The protein is thus focussed into a sharp, narrow band dependent on the value of its pI.

A stable pH gradient across a supporting medium is initially established by focussing a series of carrier ampholytes, amphoteric substances that were first synthesised in 1966 by Vesterberg and are homologues of aliphatic polyamino and polycarboxylic acids

Fig. 5.1 Net charge / pH curve for a protein

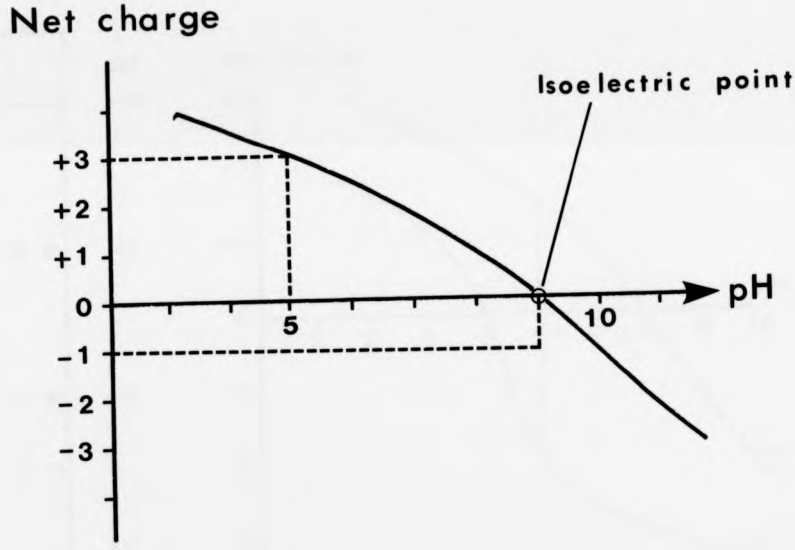
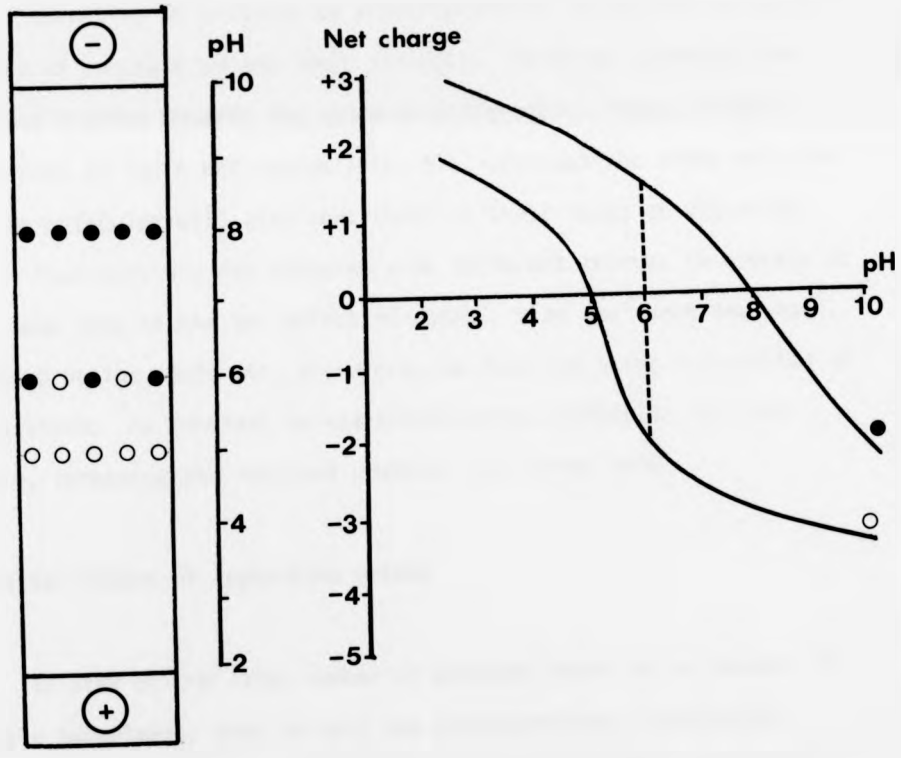


Fig.5.2 Electrofocussing - in a pH gradient



(Fig. 5.3). They possess good conductivity and their high buffering capacity imparts a pH to their environment. The conductivity of carrier ampholytes is high initially, but declines on the formation of a pH gradient, resulting in a reduction in current. Power is a product of voltage and current, thus if a constant power is used, falling current will result in rising voltage which could cause overheating of the gel support medium. An upper limit to voltage must be set to provide a safety zone, allowing a drop in current if necessary (Fig. 5.4)

2. PAGE Electrophoresis

Separation of proteins by electrophoresis is carried out in a buffer of constant pH and ionic strength. Proteins placed at the cathode migrate towards the anode at differential rates, largely according to their net charge (Fig. 5.5) although the shape and size of the molecules will also contribute to their speed of migration. It is thus possible for proteins with different charges to migrate at the same rate if the net effect of charge, size and shape are equal. These proteins would not, therefore, be resolved using this method of separation. In contrast to electrofocussing, diffusion can take place, spreading the resolved proteins into broad bands.

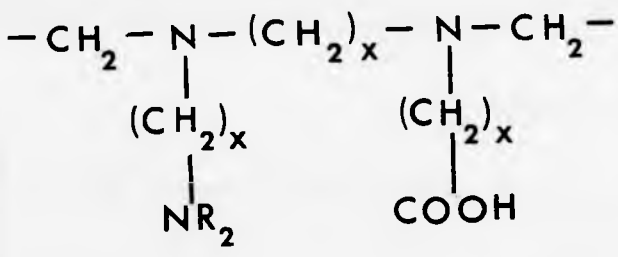
Initial choice of separation method

In view of the large number of proteins known to be present in beetle haemolymph, some in very low concentrations, isoelectric focussing was felt to be the method of choice for their separation. Agarose, a galactose polymer, was chosen as a supporting medium for the following reasons: i) it is suitable for the separation of proteins over a wide range of molecular weight, up to 2,000,000 (insect haemolymph is reported to contain conjugate proteins of

Fig. 5.3

LKB Ampholine carrier ampholytes

General formula



where R = H or $-(CH_2)_x-COOH$

X = 2 or 3

Fig. 5. 4 Constant power electrofocussing

Power = Current x Voltage

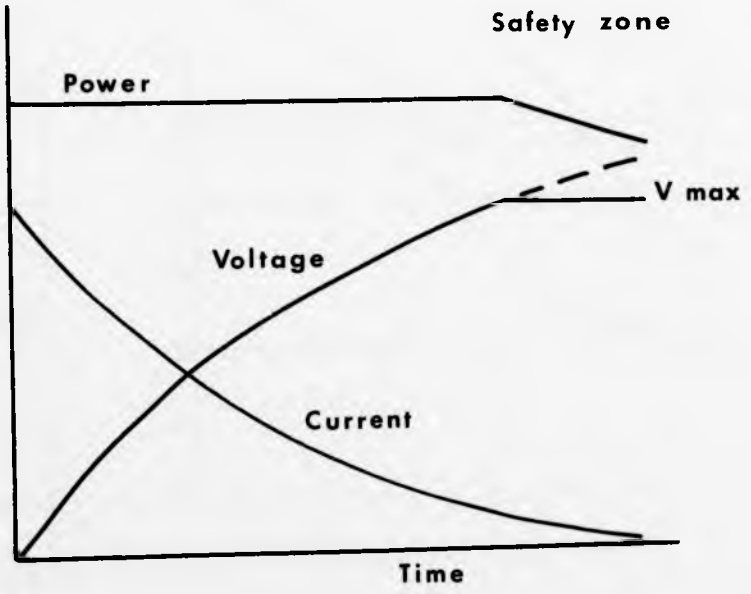
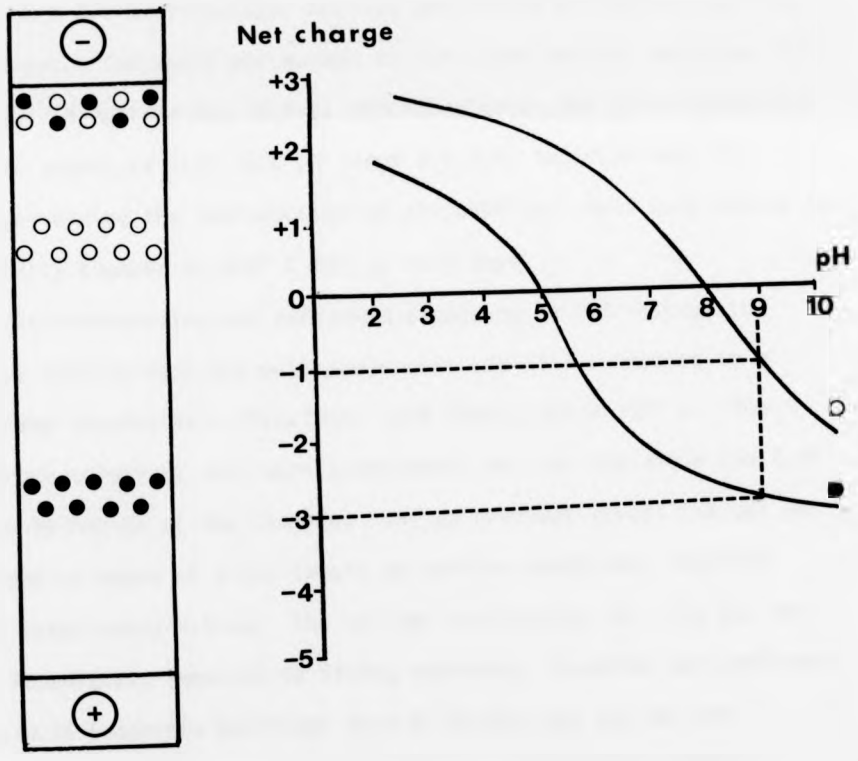


Fig. 5.5 Electrophoresis- in a constant pH



molecular weights 1-2,000,000), ii) the technique is very rapid, particularly when using thin-layer gels, iii) it is non-toxic and iv) gels can be cast on a flexible support foil which is easy to handle and store.

PART I

PROCEDURE FOR ELECTROFOCUSSING IN THIN LAYER AGAROSE GELS

A capillary gel-mould (138 mm x 125 mm) was assembled in which was cast a 0.5 mm thin-layer agarose gel, using an LKB GelBond film as support. The mould was warmed to 70° C and the gel solution (0.18 g Isogel TM Agarose EF, 16.6 ml distilled water and 1.4 ml ampholine carrier ampholyte (LKB 1818 pH range 3.5-9.5) injected into the mould, avoiding the introduction of air bubbles. Gels were stored in a humidity chamber at 2-8° C for up to 4 days.

Electrofocussing was performed according to LKB instruction booklet 1818-A, with the multiphore unit LKB 2117 connected to a Multitemp thermostatic circulator (LKB 2209), set at 10° C. The electrode solutions used were 0.5M acetic acid at the anode and 0.5M sodium hydroxide at the cathode. The pH gradient across the gel was measured by means of a Pye Ingold pH surface electrode, readings being taken every 0.5 cm. The gel was re-focussed for five min and then immediately immersed in fixing solution. Staining was performed using 0.5% Coomassie Brilliant Blue G (B1131) and the gel was destained and dried in a stream of hot air. Details of fixing, staining and destaining solutions are included in Appendix 1. The optimization of running and staining conditions is discussed below.

Development of isoelectric focussing technique

1. Running conditions

The optimum running time was ascertained by following the migration of a coloured protein, myoglobin (2mg/ml), 5 ml of myoglobin being applied to each side of the gel and the migration of these spots timed until they met at their pI. A pre-focus period of 10 min, followed by 1h focussing was subsequently used for all trials. The electrical parameters were controlled by pre-setting the voltage and current to maximum and the power to zero. At the beginning of electrophoresis, voltage was elevated to 500 V by increasing the power. Voltage had increased to 1500 V, current fallen to 10 mA and power was maintained at 10 W after an hour of focussing.

2. Staining

The timing of staining and destaining procedures was adjusted to ensure complete removal of background stain. Following 10 min immersion in fixing solution the gel was washed in ethanol to remove ampholyte. Washing time was increased to 15 min because remaining ampholytes were interfering with destaining, staining time reduced to 4 min and destaining time increased to 30 min, with one change destaining fluid.

3. Sample application

Samples of 2 µl or less could be applied directly to the gel. Samples of greater volume (10-20 µl) were applied on application pieces after first being diluted with 10 µl distilled water. Since this method of application left large areas of precipitated protein in an arc around the point of application (plate 5.1A), direct application of undiluted haemolymph was considered a better method. A volume of 2 µl was sufficient to enable both major and minor

protein constituents to be visualized without overloading the gel (plate 5.1B)

Using this method, some proteins were precipitated at the point of application. Various application positions were therefore tested to eliminate the possibility that the original position chosen coincided with the isoelectric point of some of the major haemolymph proteins (plate 5.1C). An application point 2 cm from the cathode was found to be the best position, although stainable material still remained at the application point.

4. Sample preparation

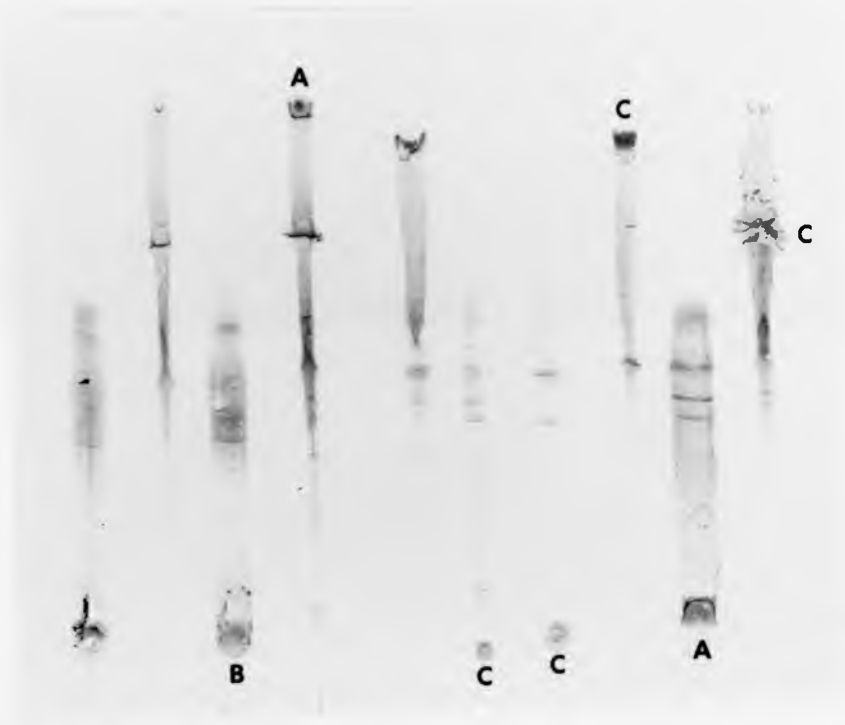
When the above parameters had been defined, separation of haemolymph proteins was still found to be indistinct, particularly in the pH range 7-8, with tailing protein zones and precipitation of protein along the track, being visible. It was felt that the method of sample preparation may have been responsible for this poor separation and this possibility was investigated as follows.

Haemolymph used for electrophoresis was prepared by the method previously described (Ch.3) and stored at -20° C. Prior to its application to the gel it was thawed and the microcap seal, together with the haemocyte plug, removed. The following variations in sample handling were tried: i) applying fresh rather than pre-frozen haemolymph, ii) a second centrifugation of samples to ensure complete removal of haemocytes and thus eliminating the possibility that cells or cell fragments were contaminating the gel, iii) addition of a crystal of phenylthiourea to prevent melanization, which was observed to occur during the sample application period, iv) addition of 1% glycine to the samples and v) pre-treatment of the samples with sodium dodecyl sulphate.

There was some improvement in band resolution when fresh haemolymph was used, but neither an increase in centrifugation time,

Plate 5.1

Haemolymph separated by isoelectrifocussing



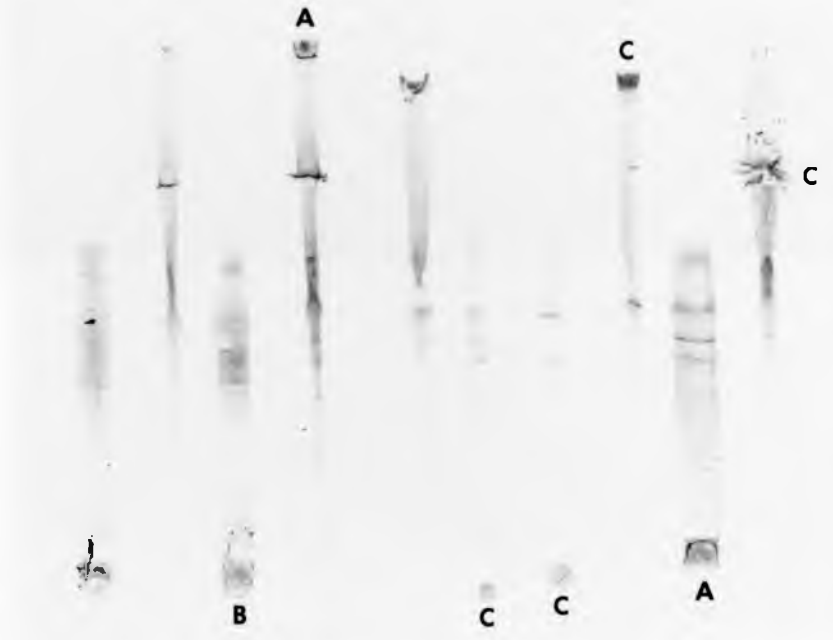
A Sample applied on a wick

B Sample applied directly to gel

C Samples applied in different positions

Plate 5.1

Haemolymph separated by isoelectrifocussing



A Sample applied on a wick

B Sample applied directly to gel

C Samples applied in different positions

addition of phenolthiourea nor dilution with 1% glycine improved separation (Plate 5.2). In order to solubilize proteins which may have been precipitated on the gel, pre-treatment of the samples by boiling for 2 min with an equal volume of 0.5M $NaHCO_3$ containing 1% SDS at pH 10 was performed. This resulted in all the proteins migrating to the anode rather than being precipitated at their isoelectric point, due to the negative charges imparted by the bound SDS molecules (Plate 5.3).

RESULTS AND DISCUSSION

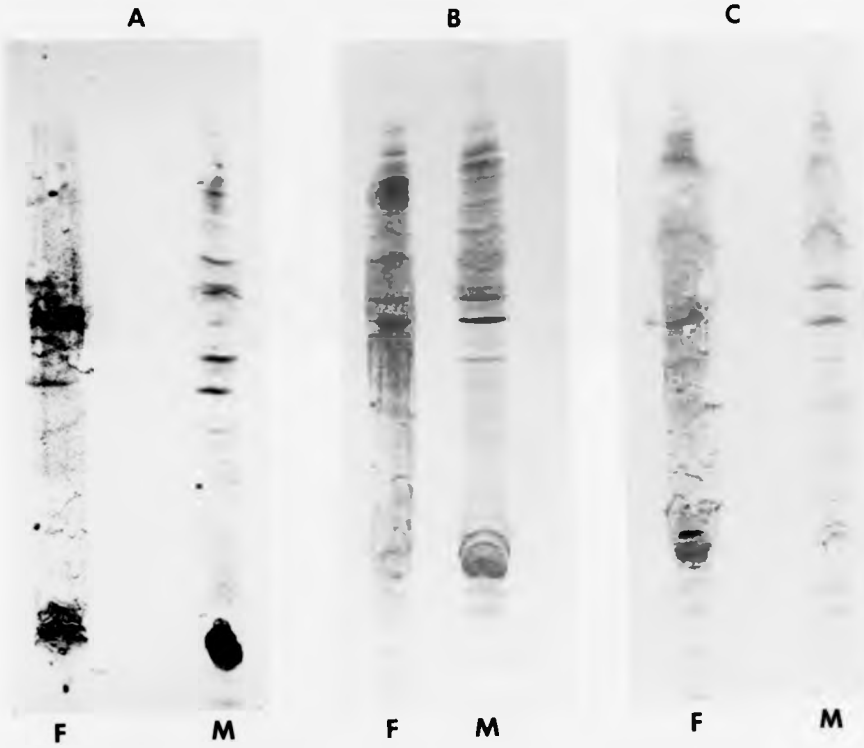
The separation achieved using the running and staining parameters described above, and applying fresh haemolymph, could not be improved upon. Haemolymph (1-2 µl) from infected and non-infected beetles was resolved with a maximum of 36 bands. Isoelectric points ranged from 4.5 - 9, the majority lying between pH 4 - 4.5. No difference could be detected between haemolymph from infected and non-infected beetles. Haemolymph from female beetles, both infected and non-infected, could not however, be fully resolved using this method (Plate 5.4).

De Loof, Lagasse and Bohyn (1972), investigating protein yolk formation in the Colorado beetle, described some of the characteristics of one of the major haemolymph proteins, vitellogenic female protein, and stated that it was very easily precipitated by $-SO_4^{--}$ containing polysaccharides. Agarose is a galactose polymer and contains free sulphate radicles.

It was therefore considered that, in common with the Colorado beetle, haemolymph from female Tenebrio molitor may possess a vitellogenic protein that is sensitive to $-SO_4^{--}$ containing polysaccharides, and that agarose is therefore, an unsuitable medium

Plate 5.2

Haemolymph subjected to various treatments prior to ISOEF



A centrifuged once - PTU added

B centrifuged twice

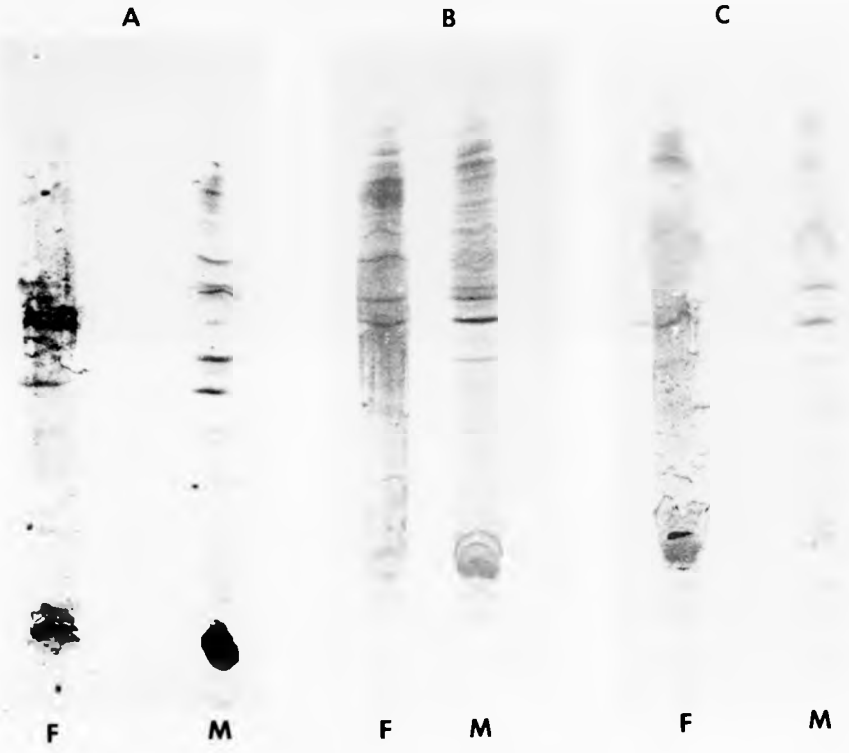
C centrifuged once - glycine added

F female

M male

Plate 5.2

Haemolymph subjected to various treatments prior to ISOEF



A centrifuged once - PTU added

B centrifuged twice

C centrifuged once - glycine added

F female

M male

Plate 5.3

Haemolymph treated with SDS prior to ISOEF

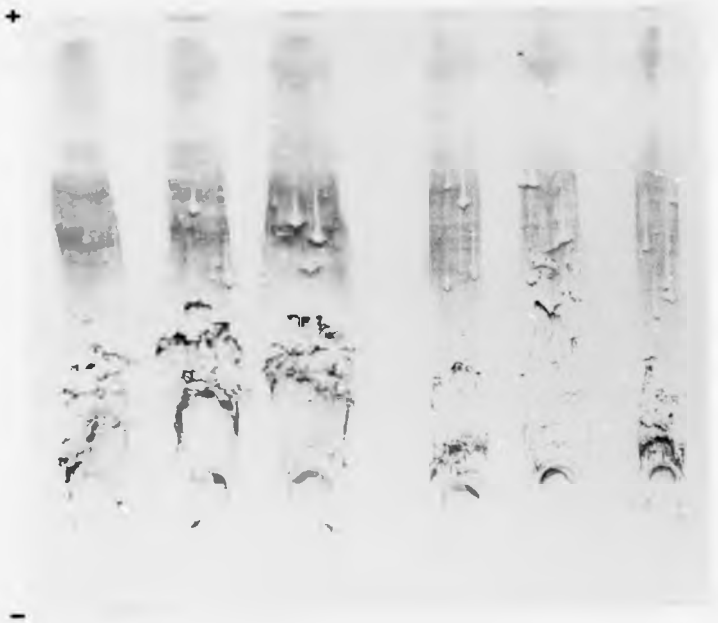
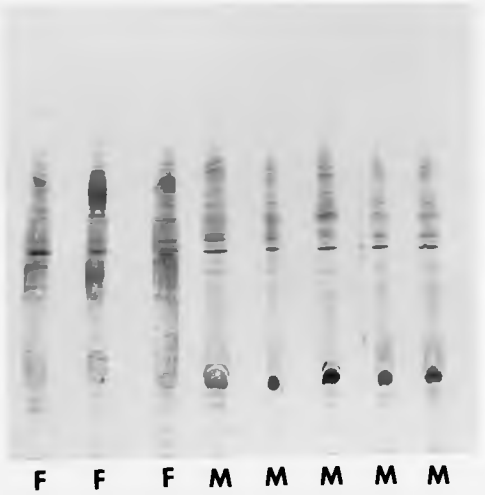


Plate 5.3

Haemolymph treated with SDS prior to ISOEF

Plate 5.4

Haemolymph separated by ISOEF



F female

M male

Plate 5.4

Haemolymph separated by ISOEF

F female

M male

in which to attempt its electrophoretic separation. The use of polyacrylamide as a supporting medium was therefore tested.

PART II

PROCEDURE FOR ELECTROFOCUSSING ON THIN LAYER POLYACRYLAMIDE GELS

Using the capillary mould described previously a 0.5 mm polyacrylamide gel was cast containing; 3.5 ml acrylamide stock solution, 3.5 ml bis-acrylamide stock solution, 1.5 ml ampholine carrier ampholyte (LKB 1818 pH range 3.5-9.5), 12 ml distilled water and 0.5 ml ammonium persulphate stock solution. Details of stock solutions are contained in Appendix 2.

One to two µl of haemolymph was collected in the usual way, centrifuged for 4 min and the cell free fluid applied directly to the gel 2 cm from the cathode. Electrofocussing was carried out according to LKB booklet 1818-P. The gel was pre-focussed for 10 min before the application of samples, and run at 10° C for 1 h with the power supply (LKB 2197) set at 2,000 volts, 50 mA and 25 W. The pH across the gel was measured by means of a surface electrode at 0.5 cm intervals and the gel then re-focussed for 5 min before fixing, staining and destaining according to LKB booklet 1818-P. Immersion of the gel in fixing solution for 1 h precipitated the proteins and washed out most of the ampholine. A 15 - 30 min soak in destaining solution removed the remainder of the ampholine and adjusted the pH of the gel to match the staining solution. Gels were stained in Coomassie Brilliant Blue G (B11311) for 10 min at 60° C and destained overnight with two changes of solution. Details of fixing, staining and destaining solutions are given in Appendix 3. The gel was left to dry overnight in a fume cupboard then covered with a thick plastic

preserving sheet.

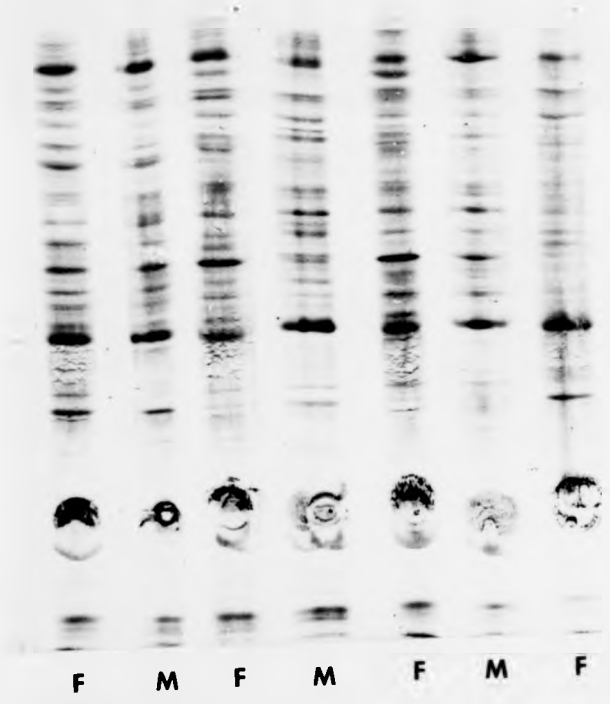
RESULTS AND DISCUSSION

Resolution of haemolymph proteins from both male and female beetles was greatly improved using polyacrylamide as a supporting medium, although streaking of female proteins still occurred to some extent in the pH range 6.5 - 7 (Plate 5.5). A maximum of 53 bands could be detected with isoelectric points ranging from pH 3.5 - 9.

Quantitative analysis of the gel profiles of infected and non-infected male and female beetles was performed using an LKB 2202 Ultrascan laser densitometer coupled to an LKB 2202 recording integrator (see Ch. 6 for details), a typical scan is shown in Fig. 5.6. As mentioned above, the area of the gel to which the sample had been applied was heavily stained, and no band resolution could be detected in this region. During densitometric analysis the integrator was disabled for the period that the laser scanned this portion of the gel and thus this artifact was not included in the calculations. Nine distinct bands were visible on every profile produced and these were used as the basis of a comparative analysis. It was found, however, that the pattern of minor bands varied between individual beetles. Peak areas of the nine consistent bands were analysed, plus the sum of peak areas between these major peaks. No proteins unique to the separate sexes were recorded and there was no consistent significant difference in peak densities when infected and non-infected beetles of either sex were examined (Tables 5.1, 5.2 and 5.3). Comparison of peak areas could only be made for samples run on the same gel as the staining and destaining procedures could not be completely standardised. Because haemolymph from female and male infected and non-infected beetles were run on the same gel, the

Plate 5.5

Haemolymph separated by PAGE ISOEF

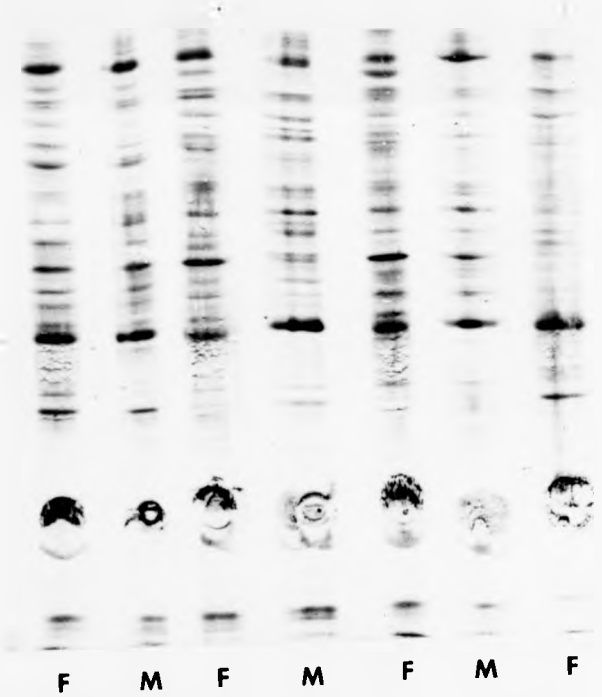


F female

M male

Plate 5.5

Haemolymph separated by PAGE ISOEF



F female

M male

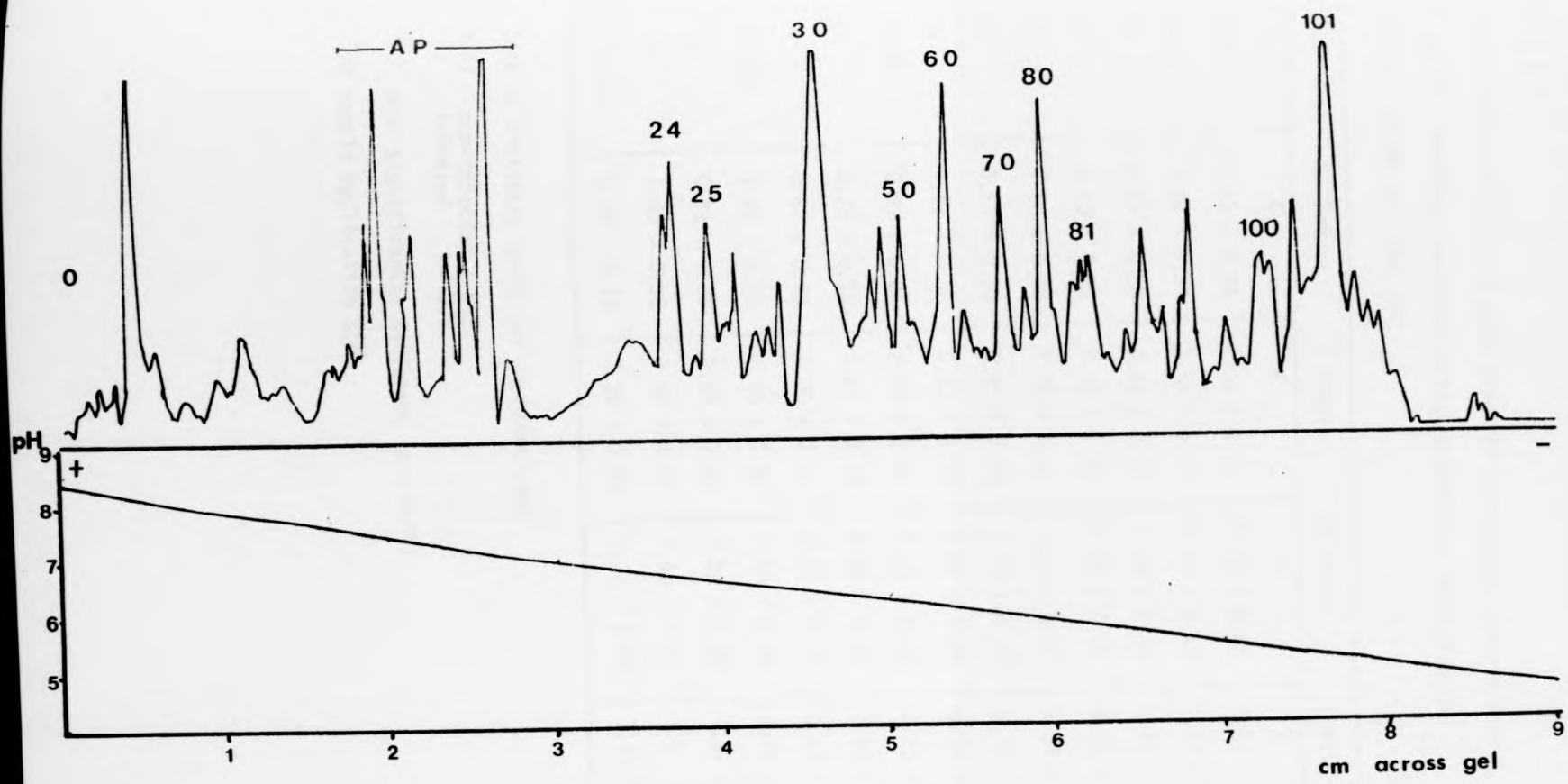


Fig. 5.6

Densitometric trace and pH profile of haemolymph separated by isoelectric focussing

AP application point

Table 5.1

Densitometric analysis of peak areas of haemolymph protein from 15 day-old beetles, separated using isoelectric focussing on a polyacrlamide gel (Gel 34)

Peak	*NI female	I female	NI male	I male
n	4	4	5	3
0-AP†	1.40 ± 0.34	2.30 ± 0.70	1.50 ± 0.40	2.80 ± 1.10
AP-30	*3.28 ± 0.30	3.43 ± 0.20	*1.90 ± 0.40	1.50 ± 0.70
24	0.37 ± 0.10	0.43 ± 0.10	0.30 ± 0.10	0.45 ± 0.10
25	0.20 ± 0.03	0.69 ± 0.30	0.23 ± 0.06	0.30 ± 0.10
30	1.16 ± 0.40	1.78 ± 0.60	1.72 ± 0.60	1.35 ± 0.40
50	0.61 ± 0.10	0.85 ± 0.20	0.75 ± 0.10	0.77 ± 0.30
60	0.51 ± 0.35	0.53 ± 0.10	0.37 ± 0.04	0.46 ± 0.20
60-80	*0.99 ± 0.07	*2.70 ± 0.60	1.03 ± 0.20	1.25 ± 0.14
80	0.78 ± 0.20	1.00 ± 0.10	0.84 ± 0.07	1.00 ± 0.20
81	0.62 ± 0.19	1.00 ± 0.10	0.52 ± 0.10	0.68 ± 0.09
81-100	1.92 ± 0.90	2.40 ± 0.50	1.80 ± 0.30	2.20 ± 0.20
100	0.82 ± 0.07	0.99 ± 0.20	1.09 ± 0.20	1.37 ± 0.30
110	1.60 ± 0.50	1.60 ± 0.50	1.40 ± 0.50	1.8 ± 0.50
110-end	*0.66 ± 0.10	1.20 ± 0.40	*2.30 ± 0.40	1.95 ± 0.30

Data in arbitrary units per µl haemolymph

* NI non-infected ± S.E.
I infected ± S.E.

** pair significantly different ($p < 0.05$)

†AP sample application spot

Table 5.2

Densitometric analysis of peak areas of haemolymph protein from 15 day-old beetles, separated using isoelectric focussing on a polyacrylamide gel (Gel 35).

Peak	*NI female	I female	NI male	I male
n	6	2	6	2
0-AP[+]	1.66 ± 0.20	2.09 ± 0.10	1.05 ± 0.18	1.89 ± 0.66
AP-30	1.67 ± 0.30	1.45 ± 0.04	0.94 ± 0.12	1.25 ± 0.30
24	0.46 ± 0.10	0.20 ± 0.14	0.25 ± 0.03	0.54 ± 0.22
25	0.27 ± 0.07	0.21 ± 0.15	0.11 ± 0.01	0.17 ± 0.06
30	1.34 ± 0.14	0.97 ± 0.14	1.17 ± 0.16	1.42 ± 0.39
50	0.44 ± 0.12	0.39 ± 0.10	0.30 ± 0.03	0.45 ± 0.02
60	0.49 ± 0.09	0.81 ± 0.02	0.32 ± 0.10	0.53 ± 0.27
60-80	*0.63 ± 0.18	*0.32 ± 0.14	0.68 ± 0.09	0.58 ± 0.41
80	0.57 ± 0.11	0.29 ± 0.04	0.45 ± 0.06	0.54 ± 0.20
81	0.52 ± 0.14	0.50 ± 0.01	0.43 ± 0.10	0.85 ± 0.16
81-100	*1.38 ± 0.26	*3.02 ± 0.24	1.05 ± 0.20	1.29 ± 0.91
100	0.53 ± 0.06	0.61 ± 0.02	0.57 ± 0.12	0.55 ± 0.10
110	0.99 ± 0.15	0.93 ± 0.04	1.01 ± 0.15	1.30 ± 0.91
110-end	0.61 ± 0.11	0.91 ± 0.70	1.05 ± 0.21	1.18 ± 0.52

Data in arbitrary units per µl haemolymph

*NI non-infected ± S.E.
I infected ± S.E.

** pair significantly different ($p < 0.05$)

[+] sample application point

Table 5.3

Densitometric analysis of peak areas of haemolymph protein from 15 day-old beetles, separated using isoelectric focussing on a polyacrylamide gel (Gel 36).

Peak	*NI female	I female	NI male	I male
n	2	4	5	5
O-AP†	4.94 ± 2.10	2.90 ± 1.50	2.84 ± 0.25	4.03 ± 0.44
AP 30	3.83 ± 1.60	3.42 ± 0.60	3.20 ± 1.50	2.50 ± 0.54
24	0.74 ± 0.04	0.68 ± 0.05	0.67 ± 0.20	0.47 ± 0.01
25	0.37 ± 0.27	0.38 ± 0.10	0.34 ± 0.30	0.32 ± 0.13
30	2.11 ± 0.32	1.08 ± 0.39	1.91 ± 0.27	1.61 ± 0.14
50	0.56 ± 0.01	0.35 ± 0.04	0.56 ± 0.11	0.42 ± 0.04
60	1.09 ± 0.10	1.00 ± 0.22	0.70 ± 0.20	0.82 ± 0.09
60-80	1.50 ± 0.43	1.04 ± 0.18	0.99 ± 0.40	1.15 ± 0.22
80	0.94 ± 0.24	0.40 ± 0.12	0.62 ± 0.10	0.55 ± 0.12
81	0.99 ± 0.14	0.64 ± 0.30	0.58 ± 0.10	0.58 ± 0.08
81-100	1.99 ± 0.2	2.58 ± 0.30	2.02 ± 0.70	1.70 ± 0.38
100	0.87 ± 0.14	0.54 ± 0.08	*0.59 ± 0.08	*1.09 ± 0.14
110	0.67 ± 0.64	0.40 ± 0.20	1.77 ± 0.19	2.04 ± 0.14
110-end	*1.22 ± 0.25	1.20 ± 0.98	*1.86 ± 0.40	2.05 ± 0.39

Data in arbitrary units per µl haemolymph

*NI non-infected ± S.E.
I infected ± S.E.

** pair significantly different ($p < 0.05$)

†AP sample application spot

sample size of each group was small. However, mean peak areas were compared from gel to gel and any trends noted. When samples from non-infected beetles were examined differences between the sexes were detected in 2 areas, namely, the area between 0 and the application point, which was denser in females, and the area after band 100, which was denser in males. Areas of elevated protein density were detected in infected females between bands 81-100 and in infected males at band 100. The area 60-80 showed an increased density with infection in female beetles on gel 34 and a decrease on gel 35 (Table 5.2 & 5.1).

Some differences have thus been detected in the density of haemolymph bands between the sexes and also between infected and non-infected beetles. It was felt, however, that due to the poor resolution of proteins, particularly in females, these results were unreliable. An alternative method of protein separation, namely PAGE electrophoresis, was therefore tried in a further attempt to elucidate the results obtained from the total protein determinations described above. This work is discussed in the following chapter.

Chapter 6

SEPARATION OF HAEMOLYMPH PROTEINS: II ELECTROPHORESIS

INTRODUCTION

A dissociating buffer system (Weber & Osborn, 1969) was chosen in an attempt to overcome the problem of protein insolubility referred to above. Sample proteins are boiled with a thiol reagent, used to cleave disulphide bonds, and an ionic detergent such as sodium dodecyl sulphate (SDS). Proteins treated in this way are denatured and the SDS binds to the polypeptide in a constant weight ratio of 1.4g SDS:1g polypeptide (Hames & Rickwood, 1981). The intrinsic charge on the sub-unit becomes insignificant compared with the negative charges of the bound detergent and migration towards the anode is a function of polypeptide size. Molecular weights can thus be determined by comparison with migration of known polypeptides.

Polyacrylamide gels have a sieving effect and the density of the gel network, or pore size, can be varied to allow separation of different sized proteins. Gel concentration is expressed as %T, where T is the total monomer per 100 ml. Total monomer includes acrylamide and a crosslinker, methelene-bis-acrylamide (Bis), which gives a covalent meshwork structure to the gel.

Electrophoresis was carried out on a horizontal thin layer gel. The procedure described in LKB booklet 306, based on the method of Weber & Osborn (1969), was followed.

MATERIALS AND METHODS

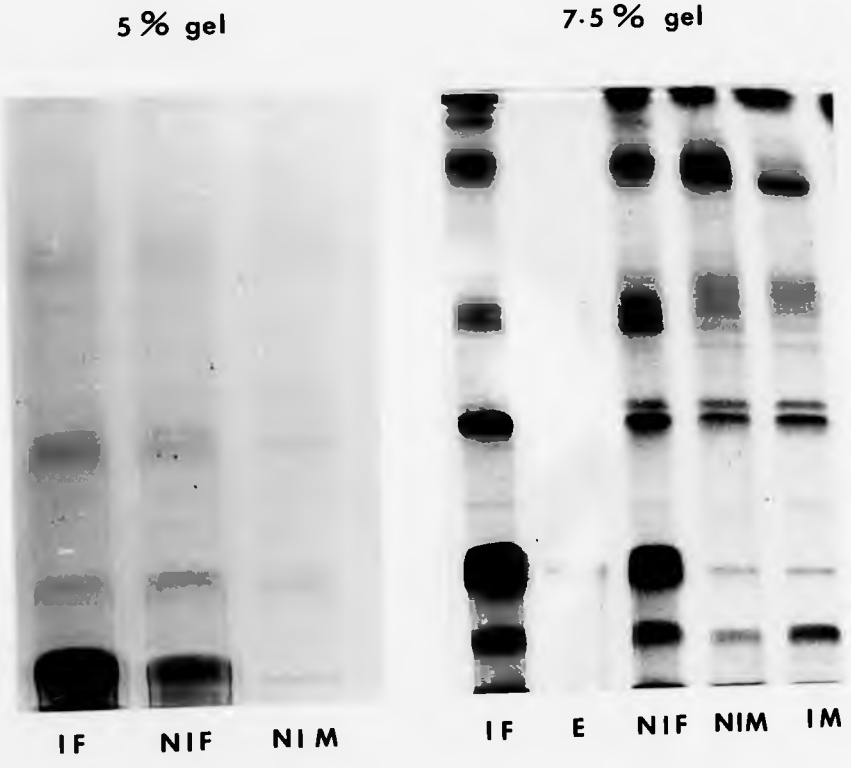
1. Preparation of the gel

All gels prepared had a crosslinker component of 2.6%. Initially 7.5% gels (T=7.5%) were cast using a gel solution consisting of: 7.5 ml distilled water, 33 ml buffer stock solution, 22.2 ml acrylamide solution and the polymerization agents: 3.2 ml ammonium persulphate and 0.1 ml N,N,N',N'-tetramethylethylenediamine (TEMED). Details of stock solutions are included in Appendix 4. The acrylamide solution was de-aerated for 30 min before addition of the polymerization agents. Gels, 2 mm in thickness, were cast in a mould 125 x 260 mm using a slot-former to produce 20 5 µl wells. After 1 h, gels were removed from the mould, wrapped in polythene and stored overnight at room temperature to ensure complete polymerization.

Three gel concentrations were used in an attempt to achieve maximum resolution of slow moving proteins (5%, 7.5% and 10%). A 5% gel was found to give good resolution whilst still being easy to handle. This gel solution was composed of: 14.9 ml distilled water, 33 ml buffer solution, 14.8 ml acrylamide, 3.2 ml ammonium persulphate and 0.1 ml TEMED. A comparison of haemolymph separation on 7.5% and 5% gels can be seen in Plate 6.1. A limited number of non-denaturing 3.5% and 5% PAGE gels were also prepared using a gel solution composed of: 14.9 ml distilled water, 33 ml Tris-glycine buffer stock solution, 22.2 ml acrylamide solution, 3.2 ml ammonium persulphate solution and 0.1 ml TEMED for a 5% gel and appropriate adjustments in volumes for a 3.5% gel.(see Appendix 5 for details of stock solutions). As can be seen in Fig. 6.1, the larger proteins from female haemolymph failed to resolve clearly and only migrated a short distance in the 5% gels. The use of a 3.5% gel improved resolution but it was extremely difficult to cast and handle.

Plate 6.1

Haemolymph separated by SDS PAGE



I infected

NI noninfected

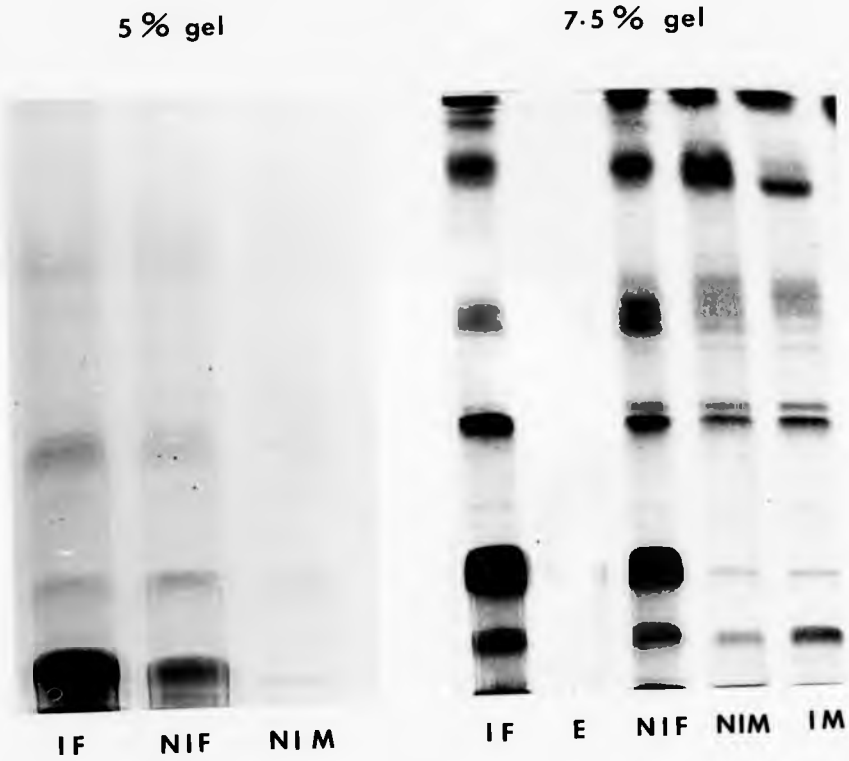
F female

M male

E egg

Plate 6.1

Haemolymph separated by SDS PAGE



I infected

NI noninfected

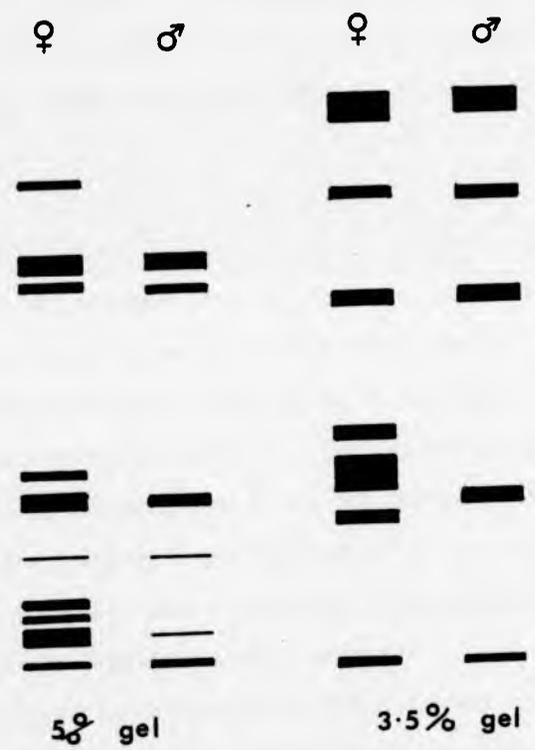
F female

M male

E egg

Fig. 6.1

Profiles of haemolymph separated by PAGE



2. Sample preparation

Haemolymph samples from 9 and 15 day-old parasitized and 9, 15 and 20 day-old non-parasitized female and male beetles were collected (Ch. 3) and stored at -20° C. Haemolymph samples were made up to 5 µl with sample buffer consisting of: 5 ml phosphate buffer (Appendix 4), 1 ml 2-mercaptoethanol and 6 g SDS in 100 ml distilled water, and boiled for 4 min. After chilling, 5 µl of bromophenol blue were added as a tracking dye. Samples were applied to the gel using a Hamilton syringe to give a haemolymph volume range of 0.5 - 1.5 µl per 1 µl well.

3. Running conditions

An electrode buffer was prepared by diluting 1 part phosphate buffer stock (0.2M, pH 7.1) with 1 part distilled water and 1.3 litres added to each of the two buffer tanks of an LKB Multiphore unit. Electrophoresis was performed at 10° C. Cooling temperatures lower than this caused some precipitation of the SDS present in the gel and buffer, which interfered with gel destaining.
Pre-electrophoresis was performed with a pre-set constant current of 150 mA for 30 min. Once the samples had been applied, electrophoresis was performed for 10 min with a 20 mA current to allow concentration of the samples on the anode side of the slot. Current was then adjusted to 200 mA and electrophoresis allowed to continue for 4 h, by which time the marker dye had migrated to the wick at the anode.

4. Staining

Details of fixing, staining and preserving fluids are given in Appendix 6. All these operations were performed at room temperature. After fixing for 1 h, gels were removed from the glass supporting

plate and stained. Staining for proteins was carried out in a solution of 0.25% Coomassie Brilliant Blue G for 2 h. Gels were destained overnight, soaked for 1 h in preserving fluid, wrapped in cellophane on a glass supporting plate and dried at room temperature.

Staining for lipoproteins was undertaken by immersing the gel overnight in a saturated solution of Sudan Black B (Gurr) in a mixture of ethanol; glycerol; water and glacial acetic acid (50: 20: 30: 5 v/v). The gel was destained for 4 days in the above solution with Sudan Black B omitted, and rehydrated in 10% acetic acid.

Glycoproteins were stained using a PAS reaction after the method of Zacharius, Zell Morrison & Woodlock (1969). Immediately after running, the gel was immersed in 12.5% trichloroacetic acid for 30 min. After rinsing lightly in distilled water, it was immersed in 1% periodic acid (in 3% acetic acid) for 50 min. The gel was then placed in distilled water on a shaking tray and left overnight to remove all traces of periodic acid. Immersion in fuchsin-sulphite for 50 min was performed in the dark and the gel then washed 3 times for 10 min each with freshly prepared 0.5% metabisulphite. Another overnight wash in distilled water on a shaking tray was performed to remove all traces of stain and the gel soaked in 5% acetic acid, covered with cellophane sheet and dried at room temperature.

5. Molecular Weight Determination

Comparison of the relative mobilities of haemolymph proteins with marker proteins of known sub-unit molecular weight enabled approximate molecular weights to be calculated. Relative mobilities, (Rm), are calculated by the division of the migration distance of the protein by the migration distance of the bromophenol blue front, compensating for any shrinkage or swelling of the gel during the staining procedures.

$$Rm = \frac{\text{distance of protein migration}}{\text{length after drying}} \times \frac{\text{length before fixation}}{\text{distance of dye migration}}$$

The Log_{10} of the molecular weight of each marker, plotted against its relative mobility, gives a straight line which can be used to interpolate the molecular weight of the samples from their relative mobilities (Fig. 6.2). A high molecular weight standard mixture from Sigma Chemical Co. was used, containing six marker proteins:

Protein	Molecular weight
carbonic anhydrase	29000
egg albumen	45000
boven serum albumen	66000
β-phosphorylase	97000
β-galactosidase	116000
myosin	205000

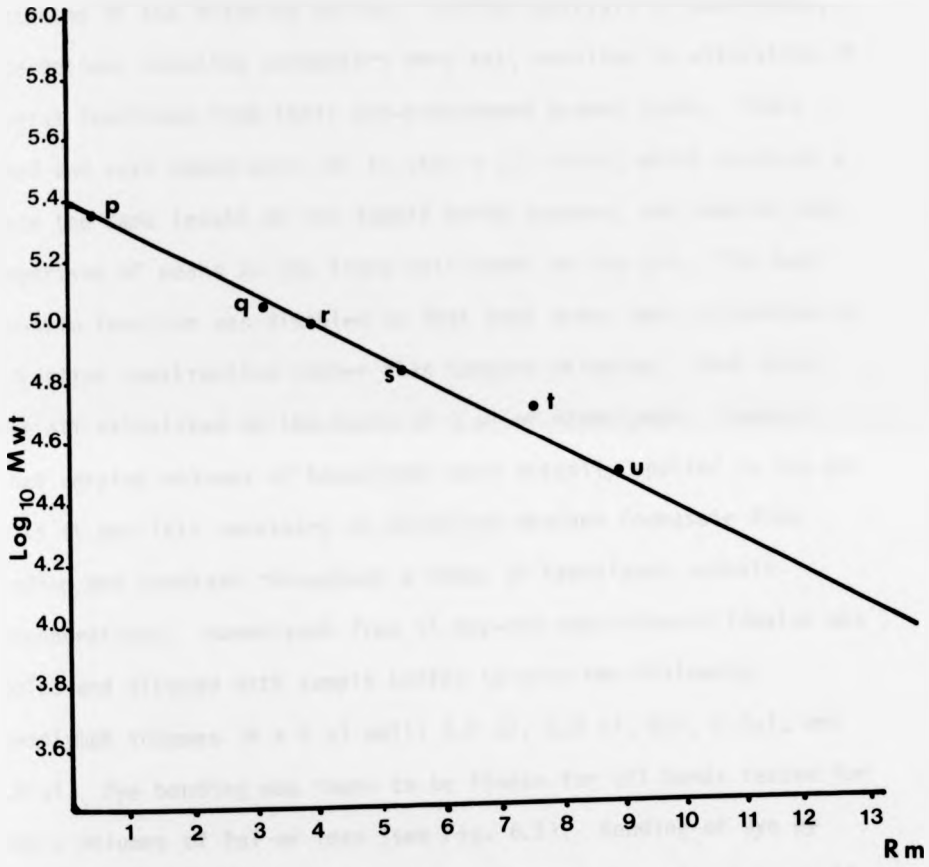
6. Quantitative analysis

Gels were scanned to obtain a quantitative comparison of band densities using an LKB 2202 Ultroscan laser densitometer, coupled to an LKB 2220 recording integrator. The laser had a fixed wavelength of 633 nm. Each sample was scanned on three tracks within the central two-thirds of the band and a mean of these readings taken. Optimum scanning parameters were established for each gel by selection of optimum scan speed, specification of the optical density range giving a full scale deflection of the largest peak, and subtraction of any background absorbance caused by incomplete destaining.

The recording integrator produced a trace of the peaks scanned by the densitometer and also a printout of peak areas calculated in

Fig. 6.2

Calibration curve for molecular weight markers



p myosin

q β-galactosidase

r β-phosphorylase

s bovine albumen

t egg albumen

u carbonic anhydrase

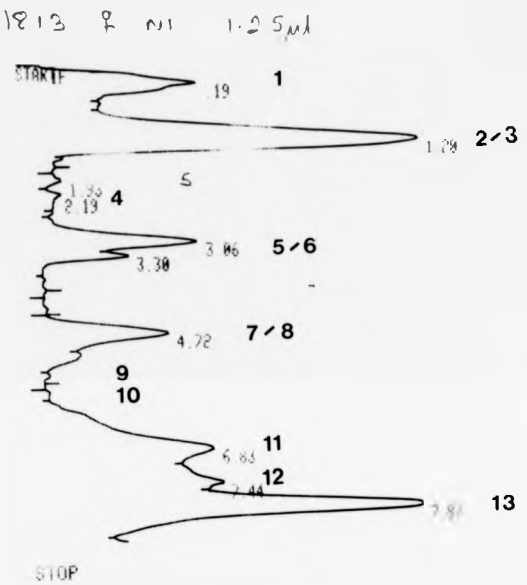
arbitrary units, area to height ratios and the peak area as a percentage of the total peaks scanned for that sample (Plate 6.2). Peaks were identified by a retention time, Rt, recorded from the beginning of the scanning period. Initial analysis of each trace, once optimum scanning parameters were set, resulted in alteration of several functions from their pre-programmed ground state. Chart speed and scan speed were set to give a 1:1 ratio, which produced a trace the same length as the sample being scanned, and enabled easy comparison of peaks on the trace with bands on the gel. The auto tan-skim function was disabled so that peak areas were calculated by a dropline construction rather than tangent skimming. Peak areas were all calculated on the basis of 1 µl of haemolymph. However, since varying volumes of haemolymph were actually applied to the gel wells it was felt necessary to establish whether Coomassie Blue binding was constant throughout a range of haemolymph protein concentrations. Haemolymph from 15 day-old non-infected females was pooled and diluted with sample buffer to give the following haemolymph volumes in a 5 µl well: 1.2 µl, 1.6 µl, 2µl, 2.4µl, and 2.8 µl. Dye bonding was found to be linear for all bands tested for sample volumes of 2µl or less (see Fig. 6.3). Bonding of dye by proteins from samples of volume in excess of 2 µl varied but was,in many cases, no longer linear. The volume of haemolymph applied to each well was thus maintained at 1-2 µl.

7. Preparation of vitellin

An attempt to identify vitellogenic proteins present in the haemolymph of female beetles was made by comparing the relative mobilities of female protein sub-units with the Rm of vitellin sub-units obtained from T. molitor eggs. Ovaries from 15 day-old females were dissected in 0.9% saline. The mature oocytes retained

Plate 6.2

Sample densitometric scan and integrator print out



RUN # 6

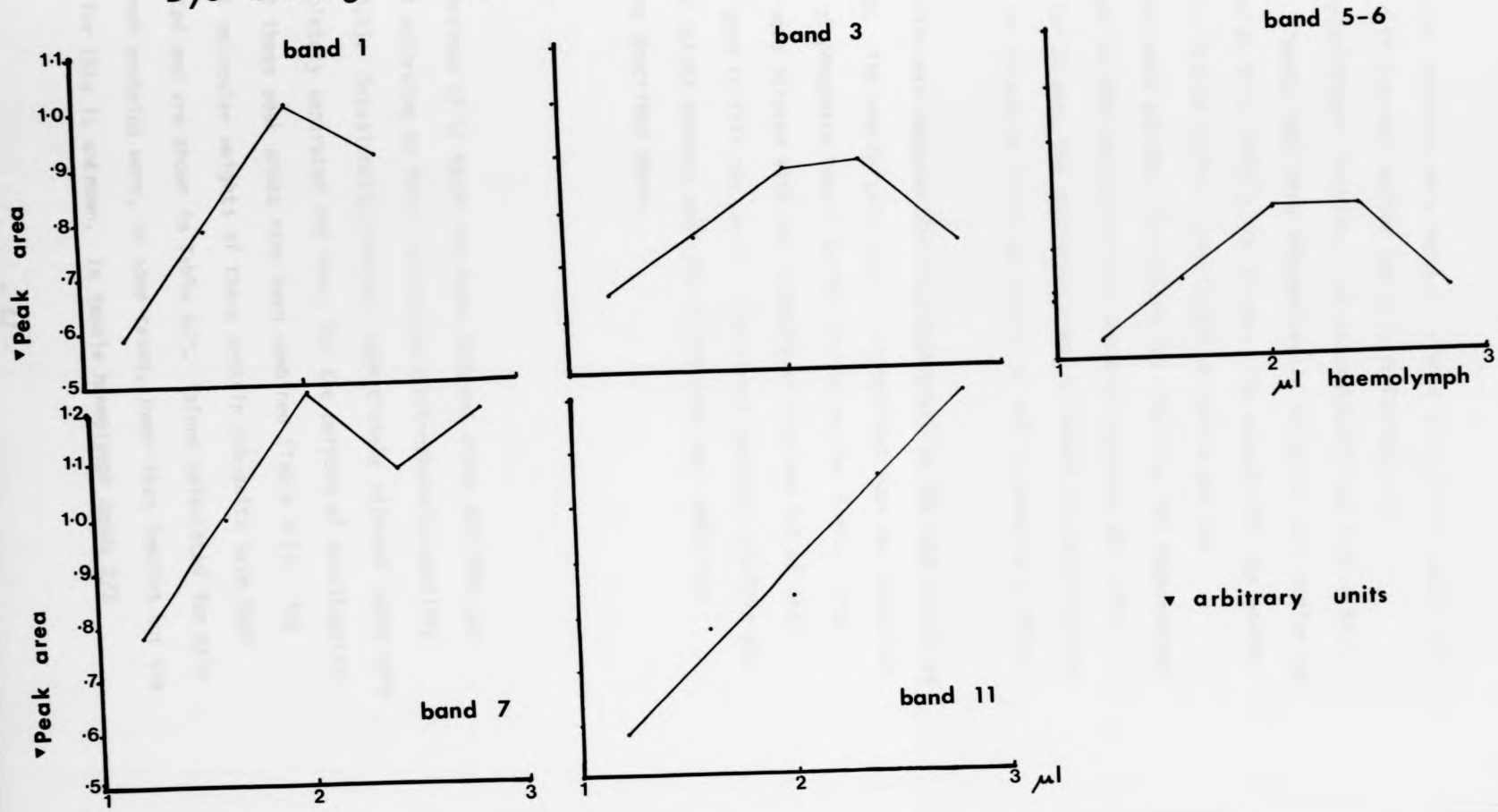
AREA% RT		AREA	TYPE	AREA	AREA%
0.19	4	0890E+07	BV	0.293	5.932
1.20	1	.8972E+08	↑ VV	0.429	2.546
1.93		2056300	D BP	0.131	0.299
2.19		4260100	PV	0.220	0.619
3.06	5	.4541E+07	VV	0.285	7.917
3.30	1	.6472E+07	D VP	0.153	2.392
4.72	5	1936E+07	PV	0.337	3.541
6.83	1	0026E+08	PV	0.501	14.557
7.4		2732E+07	VV	0.407	9.834
7.9		+08	↑ VP	0.107	23.356

TOTAL ARI 6.884E+08
1.0040E+00

1-13 see plate 6.3

Fig. 6.3

Dye binding at various concentrations



in the lateral oviducts were removed, rinsed in distilled water and stored in 0.1M Tris-HCl buffer (pH 8.1) containing 2 mM phenylmethylsulphonyl fluoride, 1 mM benzamide-HCl and 0.25 M NaCl, at -20° C. Twenty eggs were homogenized in 0.5 ml of this buffer and centrifuged at 4° C, 9000 g for 15 min. The precipitate was washed in a further 0.5 ml buffer, centrifuged as before and the supernatants were pooled. To isolate the vitellin, the supernatants were brought to 80% saturation with ammonium sulphate and, after standing for 30 min, the precipitate was collected by centrifugation (10000 g for 15 min at 4° C) and stored at -20° C (Harnish & White, 1982).

Vitellin was prepared for electrophoresis in the same manner as haemolymph. The precipitate from 20 homogenized eggs was dissolved in 20 µl of phosphate sample buffer and boiled for 4 min. Five aliquots were diluted with 5µl bromophenol blue and 5µl of this solution used to fill the wells. Haemolymph samples, vitellin and molecular weight markers were run on the same gel, under the conditions described above.

RESULTS

A maximum of 13 bands has been resolved using SDS PAGE and numbered according to their increasing electrophoretic mobility (Plate 6.3). Occasionally,however, some closely adjacent bands were not completely separated and thus, for the purpose of densitometric analysis these peak areas have been combined (Table 6.1). The apparent molecular weights of these protein sub-units have been calculated and are shown in Table 6.2. Values calculated for male haemolymph proteins were, in some cases, lower than females but the reason for this is unknown. In female haemolymph bands 2/3

Plate 6.3

A,B. Haemolymph from non-infected 15 day-old beetles, run on the same gel. A. Female; B. Male.

C,D. Haemolymph from 15 day-old female beetles run on the same gel. C. Non-infected; D. infected.

E. Egg proteins prepared by the method of Hamish and White (1982).

F. Molecular weight markers.

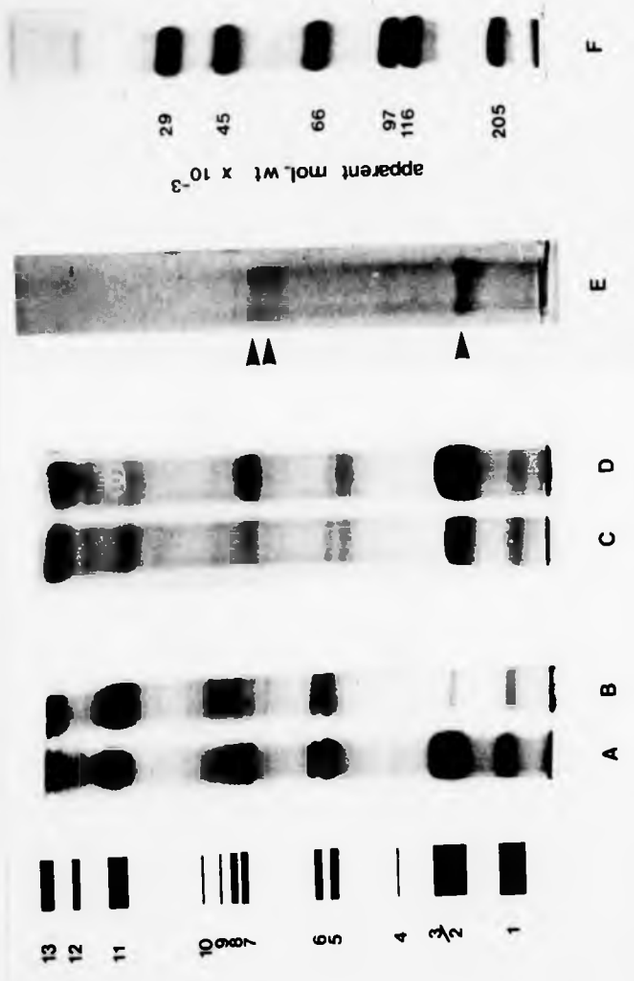


Plate 6.3 SDS PAGE profiles of haemolymph and eggs

Plate 6.3

A,B. Haemolymph from non-infected 15 day-old beetles, run on the same gel. A. Female; B. Male.

C,D. Haemolymph from 15 day-old female beetles run on the same gel. C. Non-infected; D. infected.

E. Egg proteins prepared by the method of Hamish and White (1982).

F. Molecular weight markers.

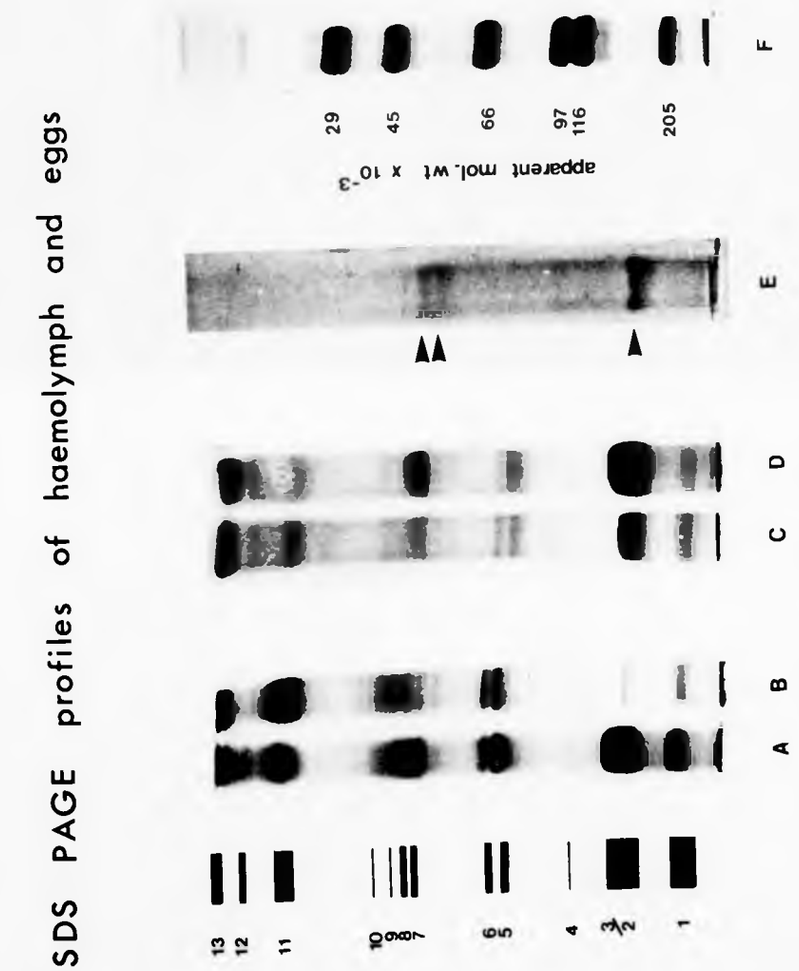


Plate 6.3 SDS PAGE profiles of haemolymph and eggs

Table 6.1 Densitometric analysis of haemolymph proteins.

peak no.	1	2/3	4	5/6	7/8	11	12	13
9 day female								
NI	1.08 ± 0.3	8.08 ± 2.1	-	2.16 ± 0.5	2.68 ± 0.5	1.62 ± 0.3	1.13 ± 0.3	3.26 ± 0.6
I	1.01 ± 0.1	7.07 ± 1.0	-	1.90 ± 0.3	2.82 ± 0.6	1.55 ± 0.5	1.11 ± 0.4	2.61 ± 0.4
15 day female		*						
NI	2.82 ± 0.7	9.64 ± 5.1	1.34 ± 0.5	5.88 ± 1.2	7.28 ± 1.4	12.23 ± 2.8	6.68 ± 1.6	21.22 ± 2.7
I	2.60 ± 0.6	16.35 ± 2.7	0.93 ± 0.3	5.19 ± 1.1	10.58 ± 1.5	12.57 ± 1.1	7.46 ± 3.0	17.49 ± 1.8
15 day male								
NI	2.41 ± 0.4	0.64 ± 0.1	0.15 ± 0.04	2.78 ± 0.3	1.17 ± 0.3	3.06 ± 0.6	-	2.71 ± 0.5
I	2.19 ± 0.4	0.69 ± 0.2	0.13 ± 0.02	2.01 ± 0.2	1.01 ± 0.3	2.09 ± 0.4	-	3.42 ± 0.5
30 day NI		*			†			
female	1.78 ± 0.8	8.27 ± 1.5	-	1.83 ± 0.2	3.90 ± 0.9	2.85 ± 0.7	1.16 ± 0.4	4.18 ± 0.7
male	1.26 ± 0.3	0.42 ± 0.1	-	1.88 ± 0.3	1.30 ± 0.3	2.03 ± 0.3	-	2.31 ± 0.4

Units are arbitrary and represent peak areas.

* p = 0.001

† p = 0.02

N = 6 or 7 in each case

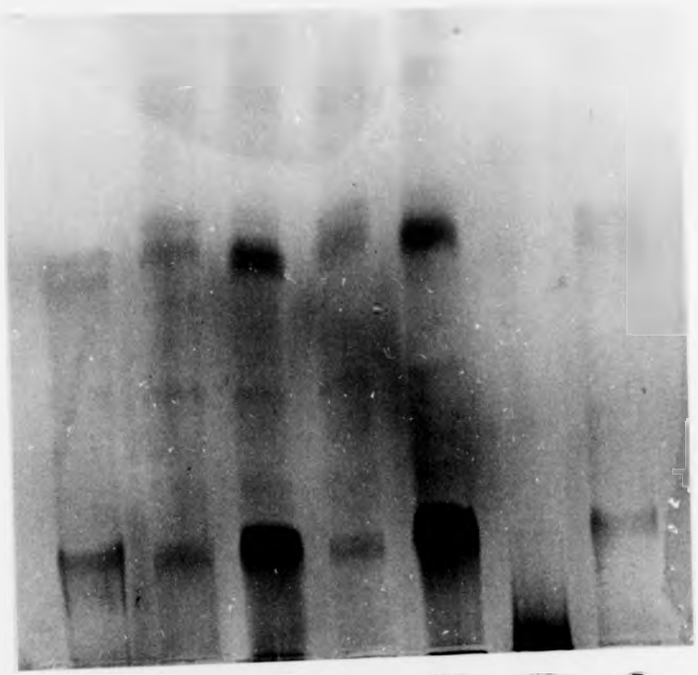
Table 6.2

Approximate molecular weights of haemolymph proteins sub-units.

Sub-unit	Female	Male	Egg
1	190,500	195,000	
2	147,900	154,800	158,500
3	144,500		
4	120,200	125,900	
5	85,100	89,100	
6	79,400	75,900	
7/8	52,500	-	56,200
9	47,800	-	53,700
10	45,800	-	
11	25,700	21,400	
12	21,400	-	
13	16,600	16,600	

Plate 6.4

Electrophoregrams stained for glycoproteins



E NIF IF E IF M E

E egg

NI noninfected

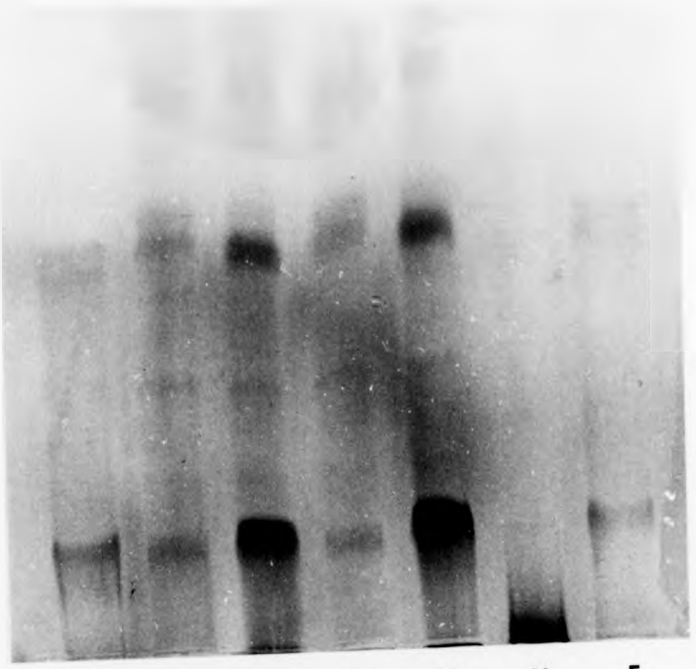
I infected

F female

M mucin

Plate 6.4

Electrophoregrams stained for glycoproteins



E egg

NI noninfected

I infected

F female

M mucin

predominated (molecular weights 147900 and 144500) and they represented approximately 37% of the total haemolymph protein. Male haemolymph contained only one component with a comparable Rm, this band, labelled 3, was a minor constituent, comprising only 5% of the total.

Quantitative comparisons of protein sub-units are shown in Table 6.1. Samples from each of the four beetle categories were run on individual gels to minimize variations that could result from experimental procedures (eg. the use of different stain batches). Comparisons cannot therefore be made between peak areas of the same bands in the four different beetle groups shown in Table 6. 1.

Haemolymph from non-infected 20 day female and male beetles was significantly different with respect to two band areas only, viz 2/3 and 7/8. Both of these were elevated in females. Staining for glycoproteins yielded a positive reaction for both of these areas (Plate 6. 4) whereas treatment with Sudan Black B revealed a lipid component only in band 2/3 in female beetles. This could not be detected in band 3 from males.

Three vitellin polypeptides were detected in egg homogenates (Plate 6. 3) and their relative mobilities corresponded to bands 2/3 and 7 and 8 in haemolymph. The approximate molecular weights of the vitellin sub-units were; 15800, 56200 and 53700. All three bands gave a positive reaction with the PAS stain for glycoproteins (Plate 6. 4).

When haemolymph from non-infected and infected beetles was compared, there were no significant differences in 9 day-old females or 15 day-old males. There was however a highly significant difference in band 2/3 from 15 day-old parasitized and non-parasitized females, peak areas from the former showing a 70% elevation (Table 6.1). Band 7/8 was also increased in parasitized 15

day-old female beetles, but not significantly so.

DISCUSSION

A comparison of haemolymph protein profiles from non-infected and infected male and female beetles was performed as a means of investigating further the recorded effect of H. diminuta metacestodes upon THSP of female hosts (Ch. 4) and to provide an explanation for the difference in protein content between the sexes.

Insect haemolymph contains a variety of proteins (Wyatt & Pan, 1978), including vitellogenins, storage and transport proteins, enzymes, hormones and proteins associated with host cellular defence reactions. Previous studies of T. molitor report differing numbers of proteins separated, which could be due to variations in the techniques employed by different authors. Thus, Wang & Patton (1968), using discontinuous gel electrophoresis, detected 21 bands in the larvae, Pembrick & Rutz (1970a) reported the presence in the adult of 18 proteins separated on a cellular acetate membrane and Laverdure (1972) detected 11 bands using "cellogel" and 15 using starch. In this study 13 bands have been separated although more than 50 are readily identifiable after iso-electric focussing (Ch. 5). As discussed in the previous chapter, the resolving powers of IEF are superior to those of EF. It is thus probable that some of the 13 identifiable bands are a composite of several sub-units, with relative mobilities too similar to allow complete separation and/or that sub-units present in low concentrations may be to diffuse to be detected as discrete bands. However, unlike IEF on agarose or polyacrylamide gels, PAGE produced no precipitate of insoluble protein nor "tailing" effects with either sex, nor did any protein fail to migrate from the application position. A quantitative

difference could be detected between some bands from non-infected and infected females and between the sexes.

A significant difference in two areas of the haemolymph profile from 20 day-old non-infected beetles has been described, namely bands 2/3 and 7/8. In the females, protein sub-units 2 and 3 were not always clearly resolved and thus, although it was possible to calculate molecular weights for each band, they were treated as one for the purpose of densitometric analysis. Male haemolymph contained a single fraction with an Rf value similar to the female band 2/3, present in significantly reduced amounts ($p=0.001$) and having no lipid component. Bands 7/8 in male haemolymph, which were also present in significantly lower concentrations ($p=0.02$), were not clearly resolved. The literature contains conflicting opinions concerning the presence of female specific proteins in T. molitor. Thus Laverdure (1972), using electrophoretic techniques, detected the presence of an extra protein fraction in haemolymph from females, 9-10 days and older, which was absent from males. She identified this protein in oocytes, and it was also detected in female pupal and adult fat body and ovary, in testis (in very low concentrations), but never in brain or mid-gut tissues. Immunological studies, however, revealed the presence of two protein fractions which reacted with anti-ovary serum, and these were also detected in reduced concentrations in male serum. Laverdure therefore concluded that the difference betwen male and female T. molitor haemolymph was quantitative rather than qualitative. Harnish & White (1982), however, prepared an antiserum against purified T. molitor vitellin, and this reacted with an antigen common to female haemolymph and crude extract, but not with male haemolymph. Clearly, without the use of a more sensitive technique, such as immunoassay, it was impossible to assertain whether the male bands 2/3 and 7/8 resolved

in this study were sub-units identical to the equivalent female bands, or whether this area was in fact composed of several bands, some being female-specific. Peferoen, Stynen & De Loof (1982) found that an examination of the protein pattern of the Colorado beetle, by a combination of electrophoretic and immunological techniques, revealed the presence of a protein with the same electrophoretic mobility as one of the two vitellogenins, but masked by it in female profiles. This protein showed no reaction with antiserum against vitellogenin and was thus clearly not a female-specific protein present in the male.

The presence of female-specific proteins in haemolymph was first reported by Telfer (1954), and subsequently they have been identified in many insect species. Hagedorn & Kunkel (1979), in a review of insect vitellogenin and vitellin, suggested that to define these proteins as female-specific was not appropriate for rigorous techniques have revealed small amounts in males. They described them as comprising 60-70% of the soluble egg yolk protein, being synthesised not in the ovary but in the fat body, and being selectively taken up by the oocyte during vitellogenesis. The existence of features which enable haemolymph soluble vitellogenin (VG) and the major yolk protein (V) to be distinguished is discussed by Hagedorn & Kunkle (1979). They state that, although no variation in amino acid or carbohydrate compositions, nor any immunological differences have been observed, findings are limited by the sensitivity of the techniques employed, and thus the distinction between the terms should be maintained. Recent reports (for example Imboden & Law, 1983) do indeed suggest that differences between V and VG do exist.

Most vitellogenins are lipoglycoproteins of high molecular weights (500K-650K), composed of a number of polypeptides of unequal

size (Englemann, 1979). There are numerous reports in the literature describing the polypeptide components of vitellogenins from a variety of insects (eg. Sams, Bell & Weaver, 1980; Peferoen, Stynen & De Loof, 1982; De Bianchi, Winter & Terra, 1982; Adams & Filipi, 1983 and Imboden & Law, 1983). Harnish & White (1982) characterized the vitellins of a number of insect species and identified three definable groups; one containing large and small polypeptides, a second group composed of large polypeptides only and a third group (the higher Diptera) with only small polypeptides. Using both non-denaturing 5% PAGE and denaturing (SDS) 7.5% PAGE they identified two female-specific haemolymph proteins in T. molitor, both containing two distinguishable size classes of polypeptides. Three bands were present in a high molecular weight class size (160K, 150K and 143K) and two bands of low molecular weight (56K and 45K). The larger class of polypeptides were present in a quantitative relationship of 3:1 with the two smaller sub-units. In this study, denaturing electrophoresis has also revealed the presence of bands (designated 2/3 and 7/8) which are found in significantly higher concentrations in females. Bands 2 and 3 have molecular weights similar to the high class size of Harnish & White (1982) and, although only one band in the area 7/8 was clearly resolved in the molecular weight determinations, this, with a molecular weight of approximatly 52K, corresponded to their small size group (Table 6.2).

Examination of the electrophoretic profile of soluble egg protein reveals that the three vitellin sub-units correspond to the bands 2/3 and 7/8 of the female haemolymph (Plate 6.3) and calculations of the molecular weights revealed these to be similar to those quoted above for T. molitor vitellogenin (Harnish & White, 1982). Thus, strong circumstantial evidence pointing to the identification of bands 2/3 and 7/8 as vitellogenins is provided by:

the determination of molecular weights, the similarity of their Rf values to those for vitellin polypeptides, their presence in significantly greater amounts in female haemolymph and the histochemical evidence presented above, which showed band 2/3 to be a lipoglycoprotein and band 7/8 to be a glycoprotein.

Densitometric analysis of gels from non-infected and infected 15 day-old females has shown that the differences in total haemolymph protein occur only within these vitellogenic fractions. No elevation in female-specific proteins was detected in 9 day-old beetles, this observation being in accord with comparisons of THSP at this age (see Ch. 4). It therefore seems reasonable to conclude that protein elevation observed as a consequence of the infection of female T. molitor with H. diminuta is entirely due to increased concentrations of vitellogenin in the haemolymph.

The synthesis and release of vitellogenins by the insect fat body and their selective sequestration by the follicular epithelial cells is under hormonal control. This will be discussed in more detail in Chs. 7 and 8 respectively. Three possible explanations of the accumulation of vitellogenin in the haemolymph of beetles harbouring metacestodes of 12 days or more present themselves: (i) an increase in the synthesis and/or release of vitellogenins by the fat body, (ii) a failure on the part of the developing oocytes to sequester the proteins or (iii) a decrease in oviposition leading to the resorption of oocytes. The fact that parasites are known to interact with host endocrine systems has been discussed in Ch. 1, but it is interesting to note here that mermithid infections of locusts result in changes in protein turnover in the host fat body, oocyte resorption and changes in vitellogenin concentration (Gordon, Webster & Hislop, 1973). The effect of H. diminuta metacestodes upon fat body protein synthesis and release, uptake of vitellogenins by the

ovary and the fecundity of T. molitor have been investigated in an attempt to provide an explanation for the pathophysiology detected in the host.

Chapter 7

FAT BODY PROTEIN SYNTHESIS AND RELEASE

INTRODUCTION

The fat body of T. molitor is a diffuse organ composed of lobes infiltrated with tracheae. The majority of the fat body lies within the abdomen, loosely connected to the ventral body wall. Groups of oenocytes are associated with the lobes of the fat body which are closely applied to the lateral body wall. The central role played by the fat body in insect metabolism has been discussed by many authors (eg. Wyatt, 1975; 1980). It is responsible for the synthesis and storage of haemolymph components, maintaining homeostasis and providing the metabolites required at various stages of development.

Shigematsu (1958) first demonstrated that the primary site of protein synthesis was the fat body, and this has since been confirmed (see Chen, 1966). De Loof & De Wilde (1970) found that the mid-gut and ovaries played no significant role in protein synthesis in L. decemlineata and Wyatt (1980), in a review of fat body protein synthesis, could find no evidence of any major haemolymph protein production elsewhere. Huybrechts & De Loof (1983) however, detected the production of vitellogenins by the ovaries of Sacophaga bullata, although the fat body provided the major source of these proteins and Coons, Tarnowski & Ourth (1982) demonstrated that in the tick Rhipicephalus sanguinius, complete immunological identity existed between vitellogenin from the haemolymph, mid-gut and fat body and vitellin from eggs. They detected the presence in female, but not male insects, of mid-gut and fat body cells with large amounts of rough endoplasmic reticulum, (RER) Golgi bodies and secretory

granules and suggested that these cells synthesised vitellogenin.

There have been many investigations into the protein metabolism of insect fat bodies, and a change in role during development has been identified. Thus, in many cases, the organ functions in a biosynthetic capacity during early larval life, changes to a storage organ as pupation approaches, and reverts to protein synthesis in the adult. Ultrastructural studies have revealed changes associated with reversal of fat body function. For example, the work of Dortland & Esch (1979)on the adult Colorado beetle, described the fat body as undergoing different paths of development depending upon the light regime. At ecdysis, the fat body cells contained large amounts of protein, stored as granules consisting of light and dark zones, part of the former being thought to be made up of urate crystals. After ecdysis some of the larval fat body persisted although the cytoplasm was renewed, RER, together with glycogen rosettes and lipid droplets appearing. In long-day ovipositing females, an increase in RER and number of mitochondria occurred and the plasma-membrane became invaginated, providing an increased area for precursor uptake and synthesised protein release. In contrast, the organelles in fat body cells from short-day females disappeared, their contents being sequestered in autophagic vacuoles, lipid storage increased and two diapause proteins were taken up from the haemolymph. Investigation of fat body protein synthesis in the adult Colorado beetle has also revealed differences related to usage (Dortland & De Kort, 1978). An examination of lysine incorporation during _in vitro_ incubation of long-day female fat bodies demonstrated a 55-fold increase by day 11, this being 4 times higher than incorporation rates detected in males.

Fluctuations in fat body protein production have been detected in adult female _T. molitor_ (Pembrick & Butz, 1970b) using autoradiography. During a 3h pulse period, they showed an increase

in tritiated leucine incorporation from emergence to day 6, unmated females having a significantly greater incorporation rate than mated females during this period. A decline on day 7 was followed by an increase on day 8 for both virgin and mated beetles, whereafter incorporation increased in mated females and decreased in virgins. No account was taken of protein secretion however, which could have occurred at greater rates in mated females and, indeed, the authors found that over a 48h pulse time incorporation increased in mated but not virgin beetles. They suggested that fat body protein synthesis occurred in a cyclic fashion, at least during the first 8 days. Fluctuations in fat body vitellogenin in Diploptera punctata were linked with stages of the first gonadotrophic cycle by Mundall, Tobe & Stay (1981). Fat bodies from virgin females contained very small quantities of vitellogenin throughout this period, whilst titres in mated females increased 3-fold once vitellogenesis had ceased on day 8. High titres were maintained during the gestation period and the next gonadotrophic cycle, although haemolymph vitellogenin concentration was negligible during gestation and fluctuated during the second cycle in a manner similar to the first.

The fluctuations in haemolymph protein detected in adult T. molitor in this study (Ch. 4), and the increase in vitellogenin concentration in female beetles 12 days post-infection (Ch. 6), could be the result of an increase in protein synthesis and/or release by the fat body.

A comparison of fat bodies from non-infected and infected females has thus been made, to determine whether wet weight, total protein content and protein synthesis and release are affected by infection.

MATERIALS AND METHODS

Unless otherwise stated, insects were dissected in 0.9% w/v sodium chloride solution. Visceral fat body adhering to the ventral body surface was gently scraped loose and collected in a Pasteur pipette. Lobes of fat body attached to the mid-gut and lateral and dorsal body walls were not used due to the difficulty experienced in the removal of adhering tissue.

1. Wet weight measurement

Visceral fat body, described above, was removed from non-infected and infected virgin beetles 9, 15 & 30 days-old and mated 30 days-old, washed in saline, blotted 3 times and placed in a preweighed container and weighed.

2. Fat body total protein determination

Visceral fat bodies, collected from 9, 12, & 15 day-old virgin and 15 day-old mated females were weighed as above and homogenised on ice in 0. 3 ml of the insect buffer used to prepare vitellin (Ch. 6). This was centrifuged at 9000 g, 4° C, for 15 min using an MSE 18 high speed centrifuge. The supernatant was added to 2 ml ice-cold 10% TCA and the precipitate washed in a further 0.3 ml insect buffer (Ch. 4) centrifuged as above and the supernatant added to the TCA. Insoluble protein was pelleted by centrifugation at 10000 g, 4° C for 15 min and the pellet washed in 2 ml ice-cold 10% TCA and recentrifuged as above. Precipitated protein was dissolved in 3.2 ml 0.5M NaOH and a Lowry protein determination performed on 1.6 ml aliquots as described in Ch. 3.

3. Fat body protein separation

Separation of fat body proteins from female and male 15 day-old non-infected beetles was performed using the technique of SDS PAGE, the running conditions and fixing, staining and destaining precedures are described in Ch. 6. Fat bodies from 4 beetles were pooled and homogenised in 0.3 ml insect buffer (Ch. 4). The homogenate was centrifuged at 9000 g, 4° C for 15 min to remove cell debris and the supernatant boiled with an equal volume of SDS sample buffer (Ch. 6). After cooling, 30 µl of bromophenol blue was added to the sample and 5 µl aliquots applied to the wells of a 5% SDS PAGE gel.

4. Preparation of saline media

Measurement of the sodium, potassium, calcium and magnesium content of T. molitor haemolymph, using atomic absorption spectrophotometry (Hurd, Brown & Arme, 1982), formed the basis for the preparation of a medium with similar cation content to that of the adult mealworm blood. This solution, Tenebrio saline A, was composed of 0.825g/100 ml NaCl (141mM) and 0.745g/100 ml KCl (42.5 mM). and was used as a solvent for ^{14}C-leucine injection solution (see below).

Further analysis of Na^+ and K^+ concentrations in Tenebrio haemolymph were performed during the course of this study using the techniques of flame photometry outlined below. Standard solutions of sodium chloride and potassium chloride were made up using double distilled water, in the range of 0.05mM to 0.2mM for NaCl and 0.1 to 0.3mM for KCl and a calibration curve constructed for each cation after measurements were taken on a Corning 100 flame photometer. Haemolymph was pooled to give 10 µl samples for sodium determination and 20 µl samples for potassium determination, centrifuged to remove haemocytes (Ch. 3) and made up to 5 ml with double distilled water.

Three samples of pooled haemolymph were prepared for each cation determination and readings compared with respective calibration curves. A second saline, Tenebrio saline B, prepared on the basis of these determinations contained NaCl, 76mM and KCl, 36mM. This was used for in vitro incubations (see below).

5. Determination of fat body synthesis and release by in vivo incubation

The injection system developed for haemolymph volume measurement (Ch. 3) was used for this work. One ml of ^{14}C-leucine (Amersham, 50µCi/ml) was evaporated to dryness under nitrogen and redissolved in 2 ml Tenebrio saline A (see above), giving a radioactive concentration of 25 µCi/ml. This solution was used to fill the injection system and 1 µl of solution, containing ·025 µCi, injected into each beetle. Beetles were maintained at 26°C with a food supply for the prescribed incubation time. Fat bodies from injected beetles were dissected in a solution of 0.9% w/v sodium chloride, washed in this solution and transfered to pre-weighed tinfoil boats. After wet weight determination they were stored frozen at -20°C. To compare the amount of leucine taken up and incorporated into protein, fat bodies from non-infected and infected beetles were homogenised on ice in 0.1 ml 0.9% w/v sodium chloride and centrifuged at 9000 g, 4°C for 10 min. The supernatant was added to 1ml ice-cold 10% TCA and centrifuged at 1000 g 4°C for 15 min, the supernatant then being added to 10 ml scintillation fluid (Ch. 3). The precipitated protein was washed in a further 1 ml ice-cold TCA centrifuged as above. A total of 3 washings were found to be sufficient to remove radioactivity from the supernatant. The precipitate was dissolved in 0.5 ml of 0.5M NaOH, neutralized in 0.5 ml 0.5M HCl and this solution added to 10 ml of scintillation fluid. Radioactivity was determined

for the TCA insoluble protein and the TCA soluble supernatant as described in Ch. 3.

The amount of ^{14}C-leucine incorporated into secreted protein during the incubation period was determined as follows: Haemolymph was collected in the normal way, centrifuged to remove haemocytes and the volume determined (Ch. 3). The haemolymph was added to 1 ml ice-cold 10% TCA and centrifuged for 15 min at 10000 g. The supernatant was washed with 1 ml ice-cold 10% TCA and recentrifuged. Washing was repeated a further 3 times, each supernatant being added to 10 ml scintillation fluid. TCA precipitated protein was dissolved in 0.5 ml 0.5M NaOH, neutralized with HCl and the radioactivity of this and the TCA soluble material determined as above.

A time course study was undertaken to ascertain the most suitable incubation peroid. One, 2, 4, and 12h periods were used to determine fat body protein synthesis and release by 15 day-old non-infected and infected female and male beetles.

6. Fat body protein synthesis and release determined by *in vitro* incubation

An incubation medium based upon measurements made of the amino acid concentration of *T. molitor* haemolymph (Ch. 10) was prepared as follows: The major haemolymph free amino acids (see Table 7. 1) were dissolved in 100 ml of *Tenebrio* saline B, the solution adjusted to pH 6.5 and sterilized by filtering through a Millipore filter, pore size 0.22 µm. Leucine was omitted from the medium and added with the radioactive leucine, the weight being adjusted to bring the total leucine concentration to 1.19 mM. One ml of ^{14}C-leucine (Amersham, 50 µCi/ml) was evaporated to dryness under nitrogen and redissolved in 10 ml of this medium. (This was stored at -20° C prior to use). Sterile specimen tubes (12-13 mm Gallenkamp) were filled with 0.5 ml

Table 7.1

In vitro incubation medium for *T. molitor* fat bodies

Constituent	mM in haemolymph	mg/100 ml
Lys	3.10	45.3
His	4.52	70.1
Arg	1.07	19.0
Thr	0.84	10.0
Ser	1.10	11.6
Glu	1.51	22.0
Gln	2.76	40.9
Pro	55.89	642.9
Gly	2.09	15.8
Ala	3.60	32.0
Val	3.28	39.0
Iso	1.03	13.0
Leu	1.19	†[15.7]
Tyr	2.70	48.9
Phe	0.59	9.9
NaCl	76	440
KCl	36	270

† a combination of labelled and unlabelled leucine

of incubation medium and maintained at 26°C with constant shaking. Insert tubes were prepared to hold fat body tissue during incubation, washing and sonication. Stainless steel gauze (0.2mm mesh diameter, G. W. Heath & Son (U.K.) Ltd. Burslem, England) was sealed to one end of 40 mm lengths of glass tube (external diameter 10 mm) with Quick-set epoxy adhesive (RS components Ltd.). These tubes fitted inside the tubes containing incubation medium, enabling the fat body tissue to float in the medium during incubation and be removed quickly, the radioactive medium draining back into the specimen tube.

Fifteen day-old non-infected and infected beetles were surface sterilized with 70% alcohol and dissected in sterile Tenebrio saline B using sterile instruments. Fat bodies from two beetles were pooled and transfered with a sterile Pasteur pipette to an insert tube. Any remaining saline was drained away and the tube placed in 0.5 ml of the radioactive incubation medium described above, and incubated for 4h.

Fat bodies were removed from the incubation medium after 4h and the medium immediately added to 3 ml ice-cold 10% TCA and left overnight at 4°C. Fat bodies, retained on the mesh of the insert tubes, were washed three times with 10 ml Tenebrio saline B, to remove traces of radioactive medium, and sonicated in 3.2 ml 0.5N NaOH using a Soniprobe (Dave Instruments Ltd.).

The sonicated solution was diluted with a further 3.2 ml 0.5N NaOH and two 1.6 ml aliquots removed for protein determination using the Lowry assay, with BSA as a standard (Ch. 4). One ml of the remaining solution was added to 10 ml scintillation fluid and the radioactivity of the fat body tissue determined.

TCA insoluble protein released into the incubation medium was pelleted by centrifugation at 1000 g, 4°C for 10 min. Six washings with ice-cold TCA were required to remove all traces of TCA soluble

radioactive fractions. The protein precipitate was dissolved in 0.5 ml Protosol (New England Nuclear) and the solution added to 4 ml scintillation fluid for radioactivity determination.

RESULTS

Fat body tissue was found to be very variable both in its extent, ranging from a few small diffuse lobes to a tissue almost filling the abdomen, and its colour, from creamy-white to grey. No relationship between colour and infection was noted, nor was a significant difference detected in fat body wet weight (Table 7.2). Data presented in this table also showed there to be no wet weight variation at the ages examined, however fat body tissue had virtually disappeared in all the 30 day-old beetles examined.

Fat body soluble protein content was not found to vary with infection in 9, 12 and 15 day-old virgins and 15 day-old mated females (Table 7.3). Infection by the metacestodes of H. diminuta does not therefore appear to alter the weight of fat body tissue, nor its protein content.

Traces from densitometric scans of fat body tissue from non-infected male and female beetles and female haemolymph, run in parallel on the same gel, are represented in Fig. 7.1. The major vitellogenic protein band 2/3 (see Ch. 6) was not detected in male fat body homogenates. However a band (x), with a similar retention time to band 2/3, was present in female tissue but represented only 1.02% of the total protein content (Fig. 7.1b). In the female haemolymph represented in the profile Fig. 7.1c, this band comprised 11.68% of the total protein. The major protein sub-unit (peak y) in female fat bodies contained 12.77% of the total protein (Fig. 7.1a) and had a molecular weight of approximatly 88000. No equivalent

Table 7.2

Wet weight determinations of fat body from T. molitor females.

Beetle age	Non-infected		Infected	
	n	mg ± S.E.	n	mg ± S.E.
9 days	15	4.0 ± 0.7	19	3.5 ± 0.5
12 days	20	4.0 ± 1.3	20	2.4 ± 0.2
15 days	15	4.5 ± 0.8	16	3.9 ± 0.5

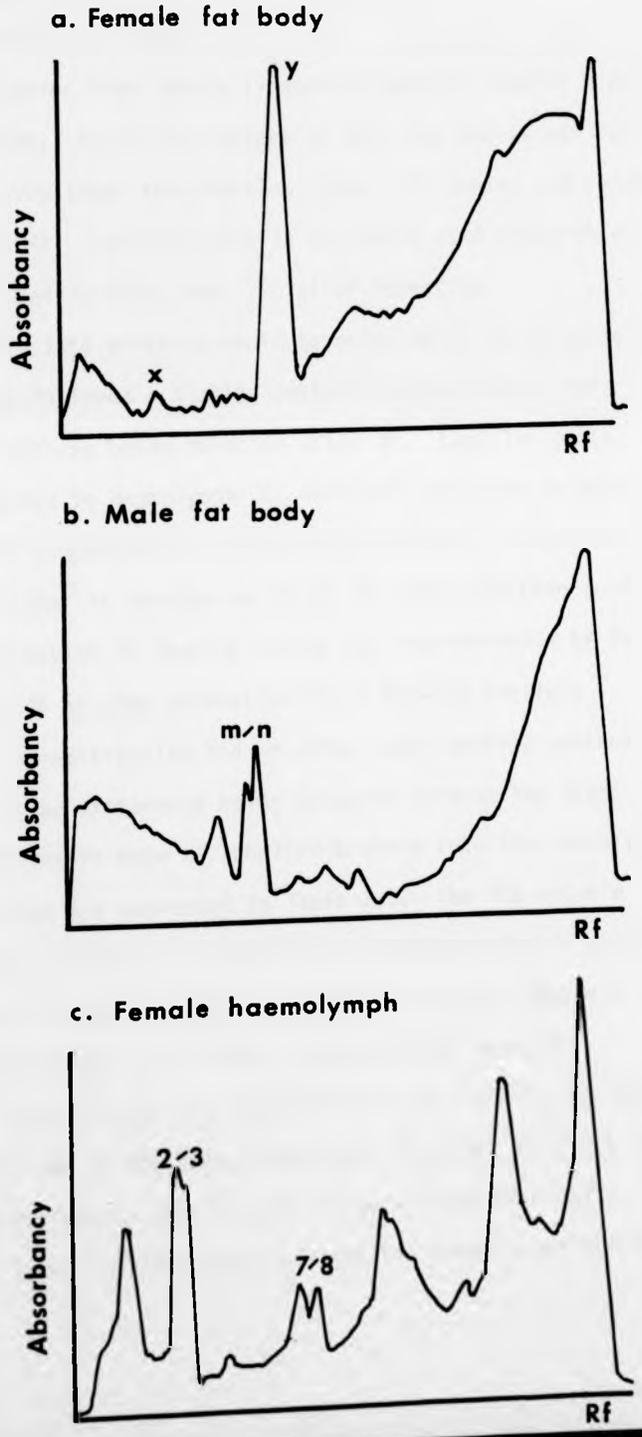
Table 7.3

Protein content of fat bodies from female T. molitor.

	µg protein per mg wet weight ± S.E.			
	n	Non-infected	n	Infected
9 day	13	65.5 ± 6.3	13	68.7 ± 2.6
12 day	8	62.9 ± 3.5	7	65.3 ± 0.1
15 day	10	52.8 ± 7.7	9	48.2 ± 4.8

Fig. 7.1

Densitometric scans of fat body and haemolymph from 15 day-old beetles



protein was detected in haemolymph samples. Male fat body tissue contained two major peaks with similar electrophoretic mobilities (m/n). Both of these had higher retention times than the female "y" protein.

Results from incubating fat bodies *in vivo* with ^{14}C-leucine for various time periods are shown in Fig. 7.2. The labelled amino acid content of fat bodies from female 15 day-old beetles reached a peak 2h after injection. Amino acid uptake by male fat bodies was found to be significantly lower than females after a 1h period and reached a peak later, at 4h. Radioactivity in the amino acid fraction of fat bodies had declined in both sexes 12h after injection. ^{14}C-incorporation into proteins could be detected 1h after injection, and its increase followed a similar pattern in both sexes (see Fig. 7.2), no increase being detected after 4h. Labelled proteins were first detected in haemolymph TCA insoluble proteins 2h after injection, their concentration rising with time (Fig. 7.2) and being significantly higher in females at 2h and 4h post-injection ($p<0.02$).

The determination of leucine uptake and incorporation by fat bodies during a 4h *in vivo* incubation in 15 day-old beetles, indicated that parasitization had no effect upon protein synthesis (see Table 7.4), no difference being detected between the sexes. Data concerning the release of labelled protein into the haemolymph during this period are expressed in Table 7.5. The TCA soluble fraction was not affected by infection and radioactivity incorporated into the protein fraction, although higher in infected beetles, did not differ significantly from that of non-infected beetles.

Results from a 4h *in vitro* incubation of fat bodies, expressed as µmol leucine per gm fat body protein are given in Table 7.6 and 7.7. Fat bodies from 15 day-old females were found to contain significantly lower concentrations of labelled protein and secreted

Fig. 7. 2

Uptake and incorporation of ^{14}C-leucine with time

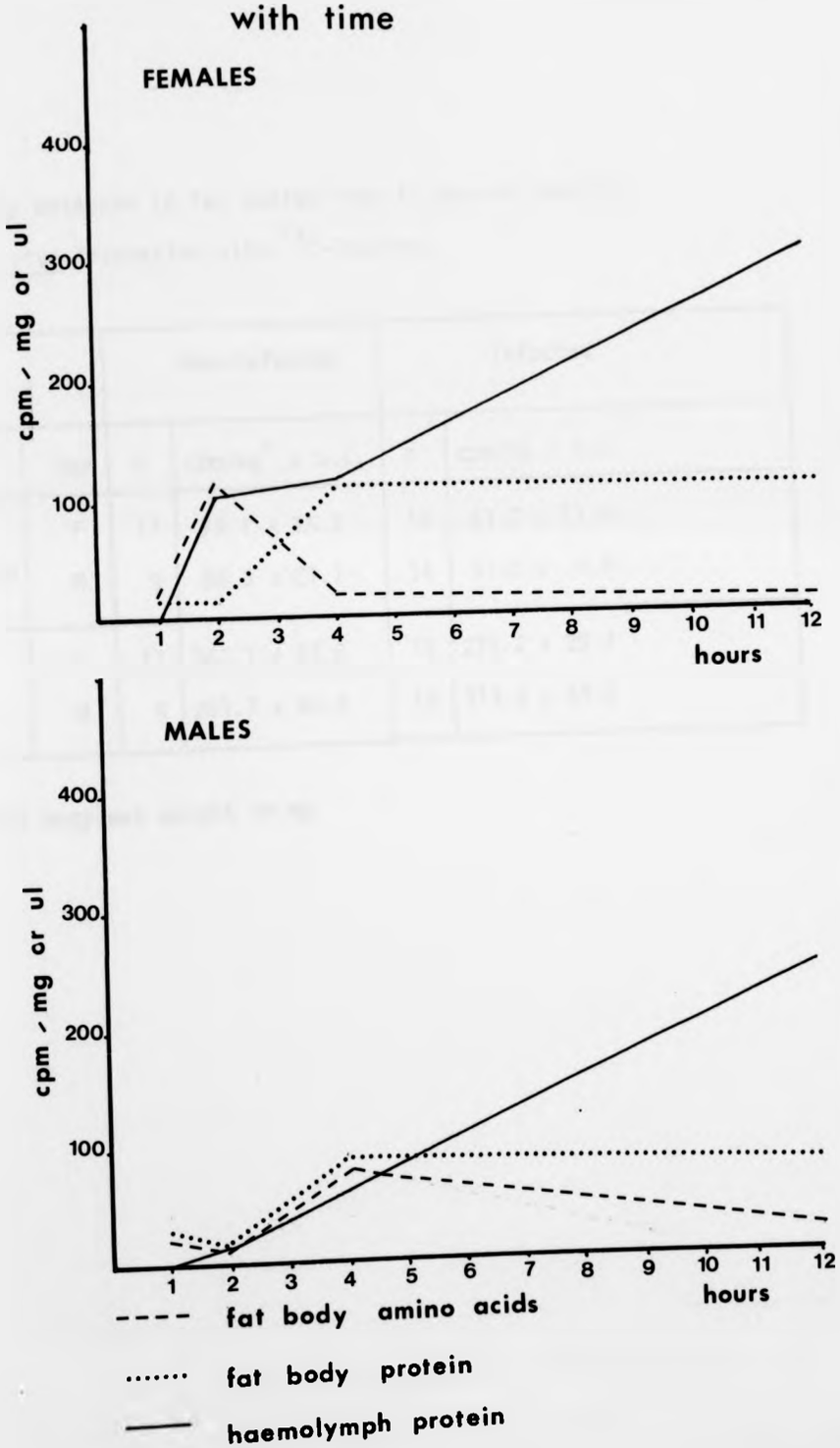


Table 7.4

Radioactivity detected in fat bodies from 15 day-old beetles after 4h *in vivo* incubation with ^{14}C-leucine.

		Non-infected		Infected	
	Sex	n	cpm/mg† ± S.E.	n	cpm/mg ± S.E.
Fat body amino acids	F	17	79.1 ± 15.1	16	61.7 ± 13.8
	M	9	66.3 ± 21.7	14	51.0 ± 4.9
Fat body proteins	F	17	363.7 ± 65.6	15	275.2 ± 29.4
	M	9	261.7 ± 49.0	14	313.4 ± 44.6

† mg = fat body wet weight in mg

Table 7.5

Radioactivity detected in haemolymph from 15 day-old females after 4h *in vivo* incubation.

	Non-infected		Infected	
	n	cpm/µl ± S.E.	n	cpm/µl ± S.E.
AMINO ACIDS	7	568.5 ± 18.7	9	556.7 ± 42.1
PROTEIN	7	343.8 ± 29.7	9	488.2 ± 85.3

Table 7.6

^{14}C-leucine content of fat bodies from 15 day-old females after 4h *in vitro* incubation.

Non-infected		Infected	
n	µMol/gm fat body protein	n	µMol/gm fat body protein
11	† 25.832 ± 6.0	11	† 9.522 ± 0.7

† Significantly different at $p < 0.02$

Table 7.7

^{14}C-leucine incorporated into fat body secreted proteins during 4h *in vitro* incubation.

Non-infected		Infected	
n	µMol/gm fat body protein	n	µMol/gm fat body protein
16	† 6.866 ± 1.27	16	† 0.514 ± 0.06

† Significantly different at $p < 0.001$

61.3% less protein into the medium.

DISCUSSION

Wyatt (1980) discussed the hypothesis, supported by evidence from several insect systems, that the synthesis of vitellogenins in the fat body initially produced high molecular-weight precursors that underwent proteolytic cleavage at some stage after translation. Thus, pulse labelling of Locusta fat body, followed by an analysis of immunospecific products led Chen, Stralhendorf & Wyatt (1978) to suggest that two large polypeptides were the initial products of separate genes and that these underwent cleavage and addition of carbohydrate and lipid within the fat body cells. Izumi & Tomino (1983) also demonstrated that separate messenger RNA coded for the two subunits of Bombyx mori vitellogenin, and that the heavy chain is cleaved post-translationally in the fat body. Sams, Bell & Weaver (1980) however reported that in Periplaneta americana vitellogenin was synthesised as a high molecular-weight precursor which was processed upon secretion.

The protein profile of T. molitor fat bodies produced in this analysis differs considerably from that of the haemolymph, particularly with respect to the vitellogenic fractions (Fig. 7.1). The absence from the female fat body of major peaks corresponding to band 2/3 or 7/8 may be due to a rapid turn-over of these proteins, very little being stored within the tissues. Alternatively, as in other insects, vitellogenic proteins in T. molitor may be synthesised as precursors and processed upon secretion. The major sub-unit "y", present only in females, is an obvious candidate for such a precursor. However, since its apparent molecular-weight of 80000 is insufficiently large to give rise, by cleavage, to the major

vitellogenin (molecular-weight greater than 144000), this is precluded. Neither band "y" in females nor "m/n" in males has been detected in beetle haemolymph and they may function as storage proteins within the fat body.

The role of adult fat body as an actively synthesising, rather than a storage, tissue has been discussed by several authors. Thus Dortland & Esch (1979) describe fat body cells of ovipositing Leptinotarsa decemlineata as highly specialized for protein synthesis, containing large amounts of rough endoplasmic reticulum (RER) and mitochondria. Vitellogenin was shown to be the primary product of female locust fat body cells (Reid & Chen, 1981). A study of the incorporation of ^{14}-C glycine into fat body proteins of the desert locust (Hill, 1965), demonstrated the establishment of an equilibrium within 1h in the fat body, activity in haemolymph proteins continuing to rise over a 4h period. In this investigation data produced by a time course study of fat body leucine incorporation showed that, after a period of 4h, there was no further increase in the amount of protein stored within the fat body (Fig. 7.2). However, the release of newly synthesised protein into the haemolymph continued for the duration of the experiment. These results support the view that the adult fat body is primarily concerned with protein synthesis. Evidence presented in the next chapter demonstrates that leucine is incorporated into secreted vitellogenins.

Results from a comparison of fat body wet weights from male and female non-infected and infected beetles indicated that parasitization had no effect upon the amount of fat body tissue present. This is in contrast to reports of metazoan infections in many other invertebrates. Gordon, Condon, Edgar & Babie (1978), for example, recorded fat body tissue autolysis and protein catabolism

following mermithid infection of simuliids. Due to the diffuse nature of this organ in Tenebrio (see above), difficulty was experienced both in extracting a constant proportion for analysis and in standardising the blotting of the tissue. Wet weight comparisons were not therefore considered a reliable means of assessing the effect of the metacestode upon host fat body.

A determination of protein content per mg wet weight, and protein synthesis during a 4h period were both chosen as more suitable techniques for this assessment. The lack of any significant difference in the protein content of fat bodies from non-infected and infected beetles at any age investigated suggests that no change is taking place in fat body protein storage in infected beetles. Thus host haemolymph metabolites utilized by the parasite do not appear to be replaced at the expense of the fat body protein reserves. This view is supported by the results from the in vivo incubation with labelled amino acid. Incorporation of ^{14}C-leucine into fat body protein reserves in both female and male beetles was not shown to differ with infection, nor was any difference detected between the sexes. Similarly the total pool of labelled amino acids in the fat body after 4h incubation was identical. The composition of this labelled pool was not analysed and it is possible that metabolism (transamination etc.) was occurring to different degrees in non-infected and infected beetles. Considerable variation in the free amino acid composition of the haemolymph has been detected between non-infected and infected beetles (see Ch. 10).

The great number of variables that exist in in vivo experiments make accurate monitoring of fat body protein synthesis difficult. The uptake and utilization of labelled amino acid by tissues such as the mid-gut has not been assessed, and proteins may be sequestered and metabolised by other organs, such as the ovaries, during this

time period. Individual variation in blood volume and fat body size also contribute to the production of a wide range of values for this *in vivo* measurement. Thus, although a greater number of counts per minute were detected in haemolymph taken from infected beetles, these figures were not found to be significantly different at a p value of 0.05. These data were very variable, analysis producing high standard errors (Table 7.5); however lack of time precluded the examination of larger samples.

Infected beetles have been shown to contain much higher concentrations of vitellogenin than their non-infected counterparts (Ch. 6). Results from these *in vivo* studies did not confirm these findings, and it was therefore decided to investigate fat body protein synthesis and release in more controlled, *in vitro* experiments.

The choice of an incubation medium in which to conduct these studies was based upon the need to approximate conditions within *Tenebrio* haemolymph. Dortland & De Kort (1978) incubated Colorado beetle fat bodies in a medium composed of *Leptinotarsa* Ringer and amino acids in the concentrations measured in whole beetles. *Leptinotarsa*, is a phytophagous beetle with a very high potassium content (Dittmer, 1981), and this is reflected in the composition of the Ringer (KCl 131mM; NaCl 2mM; $CaCl_2$ 1mM and $MgCl_2$ 5mM). Initial determination of the cation concentration of *Tenebrio* haemolymph (see above) showed the Na/K ratio to be much higher than the Colorado beetle and thus a saline (*Tenebrio* saline A) based on these measurements was used as a solvent for ^{14}C-leucine *in vivo* incubations. This initial determination was, however, based on a very small sample size and sodium concentrations were much higher than those quoted in the literature. Sodium concentrations were determined as 77mM by Butz (1957) and 66mM by Ramsay (1964) and

potassium as 33mM (Butz, 1957) and 36mM (Ramsay, 1964). Flame photometry was therefore employed to carry out a further analysis and this confirmed these reported values. Tenebrio saline B, composed of 76mM NaCl and 36mM KCl, was thus used for an in vitro incubation medium. The amino acid content of the medium was likewise based upon determinations made in the course of this study (Ch. 10). These too differed from those reported by Dortland & De Kort (1978) for the Colorado beetle, particularly with respect to the high concentrations of proline found in Tenebrio haemolymph.

The suggestion that infected beetles had a higher rate of protein synthesis was not confirmed by data from in vivo or in vitro incubations. Indeed, fat bodies from infected beetles were shown to secrete labelled protein at a significantly reduced rate ($p<0.001$) during a 4h period. This investigation also demonstrated that, within the fat body, the concentration of labelled free amino acid and of that incorporated into protein was much lower in infected beetles. The composition of these secreted proteins was not determined, therefore the amount of vitellogenin as a proportion of the total is not known. Nevertheless it would appear initially that these results are a contradiction of those obtained by protein determination and SDS PAGE for the haemolymph composition of infected and non-infected 15 day-old beetles.

The initiation and regulation of vitellogenin synthesis by juvenile hormone (JH) has been established for many species, notably in the Orthoptera (Wigglesworth, 1970; De Wilde & De Loof, 1973 and Hagedorn & Kunkle, 1979). There is mounting evidence that control of fat body synthesis in some of the Diptera is mediated by ecdysone and this is discussed in Ch. 11. Englemann, in recent reviews of vitellogenesis and vitellogenin synthesis (1979; 1980 and 1983), has identified two types of process involved in fat body protein

synthesis, one with an all-or-nothing response to varying hormone concentrations and the other a graded response. Hormonal control of transcriptional events during vitellogenin mRNA synthesis occurs in the former manner and the use of RNA-synthesis inhibitors such as α-amanitin have been shown to decrease vitellogenin synthesis in Leucophora manderae (Englemann, 1979). Associated with vitellogenin production in the fat body is the JH stimulated proliferation of the endoplasmic reticulum necessary for the large scale synthesis and export of protein (Della-Cioppa & Englemann, 1980). It has been suggested that JH enters the fat body cell and binds to a cytosol receptor (Englemann, 1980) which differs from the haemolymph JH carrier protein. Clearly vitellogenin synthesis in Leucophora involves both a JH dependent and a dose independent element determining the amount of membrane available for synthesis. Similar observations have been made for Locusta (Reid & Chen, 1981). The timing of this stimulation has been investigated in a few cases. Thus Delle-Cioppa & Englemann (1980) established the occurrence of a one-day time lag in the respones of allatectomised L. maderae to topically applied JH 111. Both ^{14}C-choline incorporation into microsomal membranes and vitellogenin biosynthesis reaching a peak 3 days after treatment, and declining thereafter.

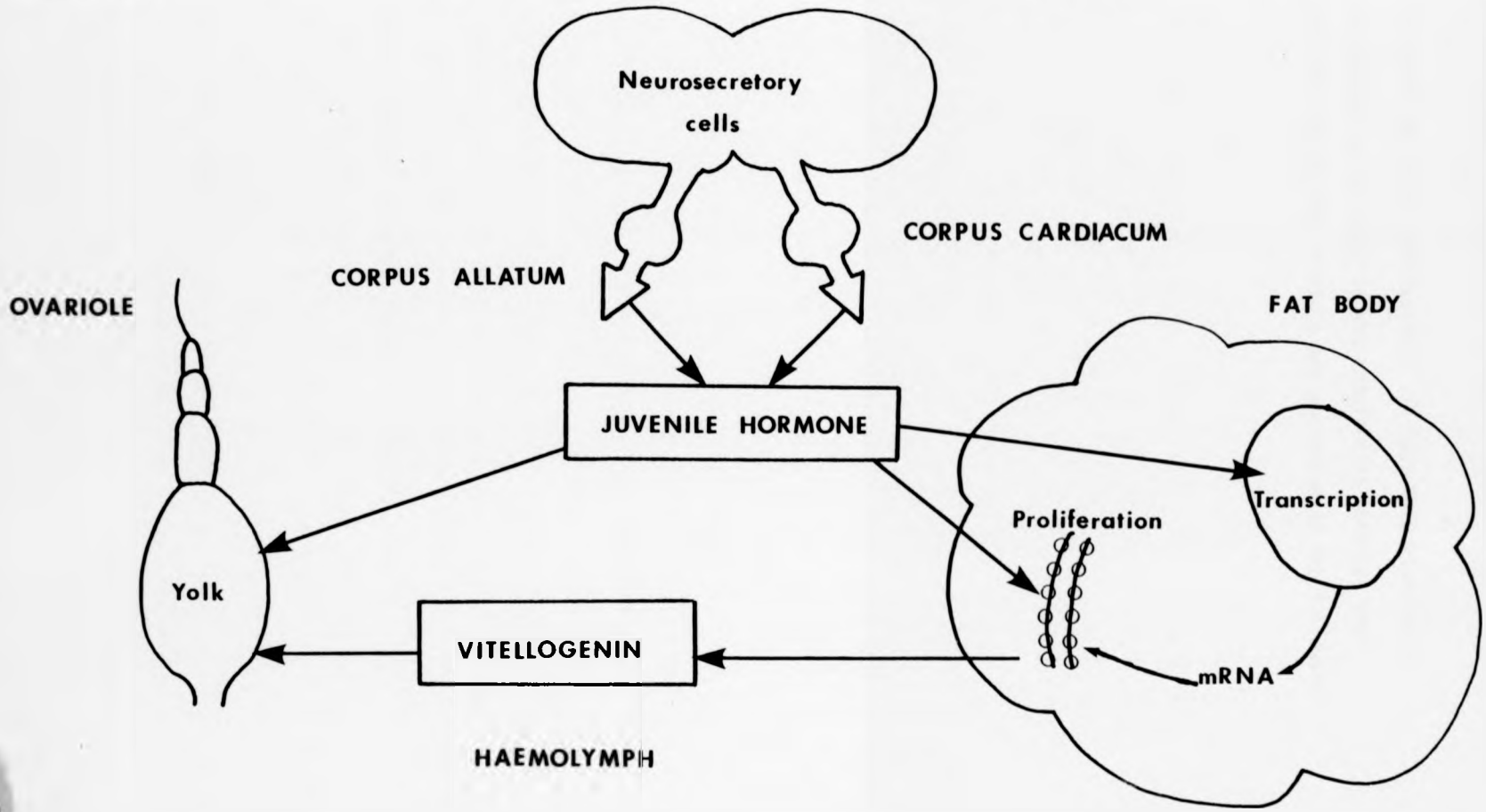
Fat body synthesis in T. molitor has been investigated by Laverdure (1972). She concluded that a product of the corpora allata was responsible for the synthesis and release of vitellogenic proteins in the fat body. In a series of allatectomy and implantation experiments it was demonstrated that events were related to the timing of this surgery. Thus, when the corpora allata were removed 48h after emergence, vitellogenesis did not cease until after the first oocytes had matured. However, if allatectomy was performed prior to emergence, no vitellogenesis occurred in the adult.

Vitellogenins were detected in pupal fat bodies and in allatectomized adult fat body for 24h after surgery. A threshold of hormonal concentration was found to be necessary to stimulate vitellogenesis, implanted larval corpora allata being more active than adult ones.

It is evident from this work that the fat bodies of newly emerged Tenebrio have been primed, possibly by exposure to JH in the larval stage, and are thus capable of secreting sufficient vitellogenin to ensure the completion of the first gonadotrophic cycle without the presence of the hormone. In this study, beetles were not exposed to H. diminuta eggs until the third day after emergence. An indirect parasite-induced reduction in vitellogenin synthesis (via JH control) may not therefore be evident during the first days of infection. Work presented in Chs. 8 & 9 demonstrates that ovaries do appear to function normally throughout the first gonadotrophic cycle.

The hypothesis that the decrease in fat body vitellogenin secretion observed in infected beetles is a result of lowered JH concentrations, provides an explanation for the discrepancy between the results presented in this chapter and those described previously. Decreased vitellogenin secretion would result in lower haemolymph concentrations in infected beetles, yet these are in fact significantly elevated 12 days or more post-infection when compared to non-infected beetles. Juvenile hormone also has a role to play in insect vitellogenesis, its concentration governing sequestration by the ovaries. This will be discussed in detail in the next chapter and its action in conjunction with vitellogenin synthesis and vitellogenesis is summarised in Fig. 7.3. Clearly, decreased hormone concentration would lead to the uptake of smaller amounts of vitellogenin and this could result in the elevated concentrations detected in haemolymph from infected females, despite the fact that

Fig. 7.3 The dynamics of vitellogenesis (simplified)



less vitellogenin was being synthesised. To test this hypothesis ovariole sequestration of proteins was examined in infected and non-infected beetles and the findings are described in the following chapter.

Chapter 8

THE EFFECT OF INFECTION UPON THE OVARIES OF T.MOLITOR

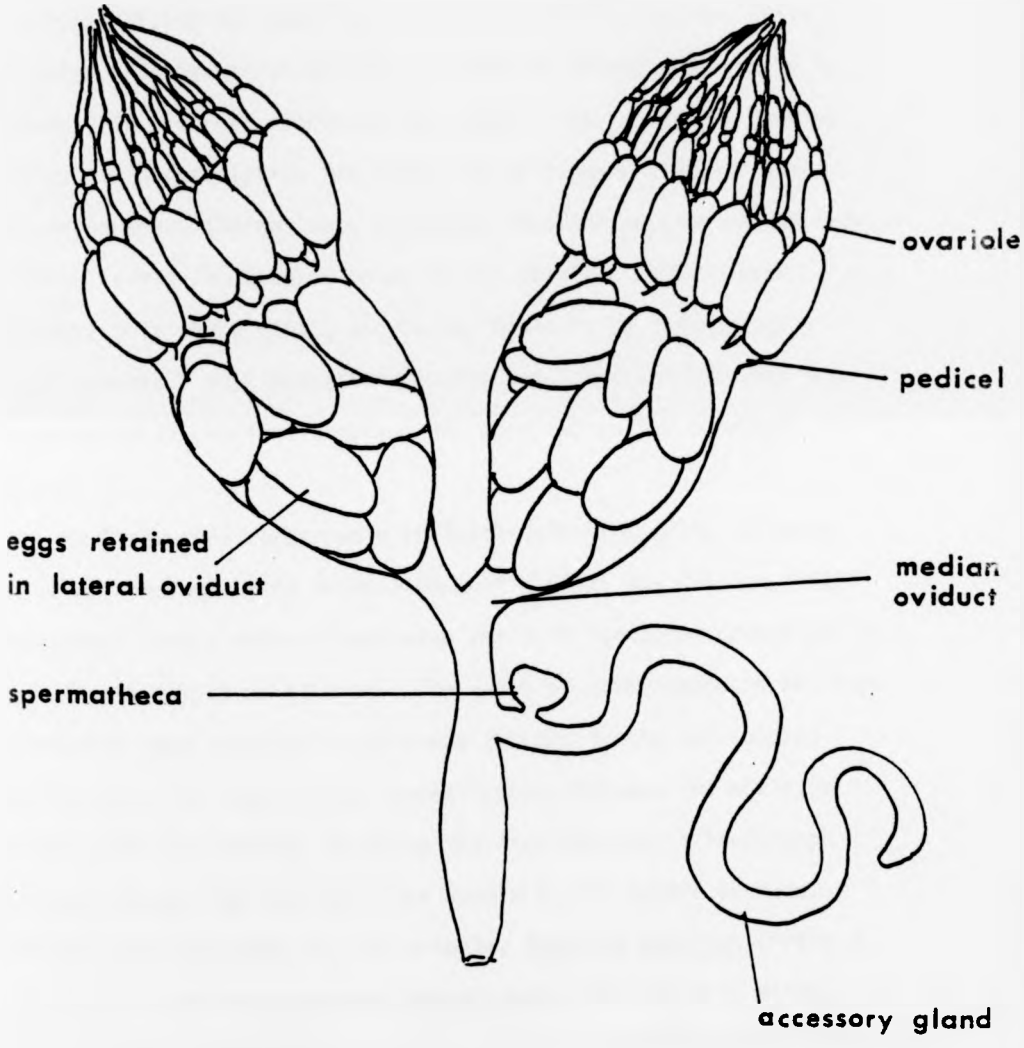
INTRODUCTION

The elevated vitellogenin concentration detected in T. molitor females, 12 days or more post-infection (Ch. 6), has been discussed in relation to its synthesis and secretion by the fat body. The work described in this section examines the hypothesis that a decrease in the ovarian sequestration of these proteins results in their accumulation in the haemolymph.

The paired ovaries of T. molitor consist of ovarioles of the meroistic type characteristic of the Endopterygota, each germarium containing oogonia, primary oocytes, nutritive or trophocyte cells and prefollicular tissue. (De Wilde & De Loof, 1973a; Englemann, 1970). Two types of meroistic ovarioles were recognised by Grosz (1903), those of T. molitor being of the telotrophic variety in which the trophocytes are aggregated in the germarium and connected to developing oocytes via cytoplasmic strands. Schlottman & Bonhag (1956) stated that each ovary contained 12 ovarioles, although other authors have found this to be more variable (see Ch. 9). They described each ovariole as consisting of an apical terminal filament (these fused to form a suspensory ligament attached dorsally to the thorax), a germarium, a vitallarium containing 3-4 follicles and a pedicel. The pedicels of each ovariole opened into a lateral oviduct, the paired oviducts uniting to form a median oviduct. (see Fig. 8.1) An outer epithelial sheath formed a continuous layer of cells from filament to pedicel, whereas the inner sheath was only evident as groups of cells along the wall of the germarium and in the constrictions between follicles. Once part of the vitellarium, the

Fig. 8.1

T. molitor female reproductive system



nucleus of the developing oocyte enlarged to become a germinal vesicle.

Follicular epithelial cells, surrounding the enlarging oocyte in a monolayer, were found initially to undergo mitosis. When the ability to divide had been lost in advanced follicles, the cells underwent a change in shape from columnar to cuboid and finally to squamous, in order to alleviate the pressure caused by the growing oocyte. This description was confirmed by Ullmann (1973). Using autoradiography, she was able to demonstrate the passage of RNA from nurse cells *via* the trophic cords to the oocytes. Within each ovariole, only the ultimate oocyte was found to be undergoing vitellogenesis. Yolk deposition in the penultimate oocyte was not initiated until chorion formation had occurred in the terminal oocyte.

The literature concerned with insect vitellogenesis is large, and recent reviews by De Wilde & De Loof (1973a) and Bradley (1983) demonstrate that a general mechanism for yolk formation cannot yet be derived from the cases studied. The onset of vitellogenesis has been observed in many insects to accompany changes in the surrounding follicular epithelium. Large spaces develop between the cells, a process known as patency, allowing the free passage of haemolymph proteins through the intercellular spaces to the oocyte surface. This has been described in, for example, *Rhodnius prolixus* (Pratt & Davey, 1972a) and *Leptinotarsus decemlineata* (De Loof & De Wilde, 1970b). These spaces can be visualized by the immersion of ovarioles in Evans Blue dye which penetrates the interfollicular spaces but does not stain living cells (Pratt & Davey, 1972a). Patency has been demonstrated to be a reversible phenomenon (Davey & Huebner, 1974, Huebner & Injeyan, 1980 & Davey, 1981) under the control of juvenile hormone (see below).

The microvillous oolemma has been shown to become pinocytotically active at the onset of patency, haemolymph proteins being taken up in clathrin-coated vesicles which coalesce to form larger vesicles, yolk granules being permanantly surrounded by membrane (Hagedorn & Kunkle, 1979 & Bradley, 1983). The highly selective uptake of extra-ovarian vitellogenin, resulting in concentrations 20-200 times higher than in the haemolymph, has been demonstrated in several insects for example, the locust (Lange & Loughton, 1981) and Cecropia moths (Anderson & Telfer, 1970). The mechanism for this selective uptake is not fully understood, but it appears that the role of the follicle cells is important as no uptake occurs in naked oocytes (Hagedorn & Kunkel, 1979). Anderson & Telfer (1970) suggested that a binding agent, secreted by the follicle cells of Cecropia moths, caused aggregation of vitellogenic proteins which were, however, labile enough to break apart during pinocytosis. A similar process has been described in the Colorado beetle by De Loof, Lagesse & Rohyn (1972). They identified two proteins which were selectively absorbed by the oocyte, a female vitellogenic protein and a chromoprotein; both were high molecular weight proteins precipitating readily in the presence of polysaccharides containing $-SO_4$ groups. Follicle cells were shown to secrete vesicles containing mucopolysaccharides and it was proposed that the contents of these secretory vesicles are released in the space between the follicle cells and oocyte surface, causing the two sensitive proteins mentioned above to be precipitated, and thus taken up more readily by pinocytosis. The presence of an intercellular matrix which binds, and thereby concentrates, vitellogenin has also been proposed for the silkmoth Hyalophora cecropia (Telfer, 1979). The occurrance of selective binding at the oolemma rather than concentration in the interfollicular spaces has also been discussed. Aldridge, Bowers &

Feldlaufer (1981) suggested that a polysaccharide recognition moiety was involved in sequestration in the milkweed bug and Ferenz, Lubzens & Glass (1981) proposed that vitellogenin is bound to the oocyte surface, and discussed the involvement of a diglyceride carrier-protein. De Loof (1983), examining the bioelectric aspects of the insect meroistic ovary, proposed that it acts as an electrophoresis chamber. He suggested that ion-pumps generate an electric current which crosses the follicles and drives vitellogenin to the oocyte surface.

Once vitellogenesis is completed, cells of the follicular epithelium secrete the vitelline membrane and a multilayered chorion and, after the egg has left the ovariole, degenerate.

The accumulation of vitellogenic proteins in the ovaries of non-infected and infected beetles has been examined and a comparison made of the dry weight of the respective tissues. The results of these studies are reported below.

MATERIALS AND METHODS

All beetles used for these studies were reared, infected and maintained as described previously (Ch. 2). Mated females were kept as single pairs without the addition of apple to their diet.

1. Ovary dry weight determinations

Ovaries from 9 and 15 day-old virgin beetles and 15 and 30 day-old mated beetles were dissected in 0.9% w/v sodium chloride solution, The lateral oviducts containing retained eggs, were severed along the line of the pedicels (Fig. 8.1) and the ovarioles washed in saline, placed in pre-weighed foil dishes and dried to a constant weight in an oven at 40° C.

2. *In vivo* incubation of ovaries with ^{14}C-leucine

Using the procedures described in Chs. 3 and 7, 9, 12 and 15 day-old female beetles were injected with ^{14}C-leucine (50 µCi/ml) and left for a 4h incubation period. Ovaries were dissected in 0.9% w/v sodium chloride, washed 3 times in 10 ml saline, blotted and placed on a pre-weighed foil dish for wet weight determination and then stored at -20° C overnight. The thawed tissue was homogenized on ice in 0.1ml 0.9% sodium chloride and determination of the incorporation of radiolabelled amino acids and TCA insoluble protein performed as described in Ch. 7.

3. Identification of the radiolabelled fraction of *in vivo* incubated ovaries

Fifteen day-old non-infected beetles were injected with 2µl of stock ^{14}C-leucine (50 µCi/ml) and maintained for 4h as described in the preceeding chapter. Ovaries were removed, washed and stored as above. Ovaries from 5 beetles were pooled and homogenized on ice in 0.05ml insect buffer (Ch.6). The homogenizer was washed in 0. 05ml insect buffer and the homogenate and washing pooled and centrifuged at 9000 g (4°C) for 15 min to remove cell debris. Thirty µl of supernatant was added to 20 µl sample buffer (see SDS PAGE Ch. 6) and boiled for 4 min. To avoid further dilution no tracking dye was added to this solution. A 5% SDS PAGE gel was cast containing 10 µl wells (Ch. 6) and 10 µl aliquots of the radiolabelled ovary solution applied to the gel in two adjacent tracks on each half of the gel. Samples from 4 groups of pooled ovaries were separated on the same gel. Electrophoresis was performed for 4h at 10° C under conditions described in Chapter 6, after which the gel was fixed for 1h then cut in half. One section was stained in the usual way (Ch. 6), and tracks from the other half of the gel were sliced into 2mm-wide

pieces, the parallel pieces from the adjacent wells (ie. from same supernatant solution) pooled and each pair incubated overnight in 0.4 ml Protosol (New England Nuclear) at 32°C, to solubilize the protein fixed within the gel. Four ml of scintillation fluid was added and the radioactivity of the protein contained in each pair of gel slices determined (Ch. 3). Counts per minute minus background for each pair of 2mm sections were plotted against gel length, and the resultant distribution compared with an electrophoretic profile of that sample, obtained from the stained portion of the gel. A densitometric scan of the stained tracks was also obtained (Ch. 6).

RESULTS

Data concerning the dry weight measurements of ovaries from non-infected and infected females are shown in Table 8. 1. No difference in dry weight was detected at any age investigated in virgin or mated beetles.

Uptake of radiolabelled amino acids and proteins from 15 day-old beetles was calculated in a pilot study on the basis of counts per minute per pair of ovaries. No difference was detected in the amino acid uptake by ovaries from non-infected and infected females during 4h *in vivo* incubation, the label incorporated into protein was however 43.5% lower in ovaries from infected beetles (Fig. 8.2). These results were confirmed by subsequent incubations, results being calculated on the basis of counts per minute per mg ovary tissue (Table 8.2). The parasite had no affect upon the uptake of labelled amino acid at any age investigated, similarly the amount of radioactivity detected in ovarian proteins was not affected by infection in 9 day-old females and the depression detected in 12 day-old infected beetles was not significant at a p value of 0.05.

Table 8.1 Dry weight measurements of ovaries from non-infected and infected T. molitor.

A VIRGIN BEETLES

Dry weight in mg ± S.E.

Beetle age	n	Non-infected	n	Infected
9 days	10	1.06 ± 0.14	10	1.62 ± 0.21
15 days	10	0.76 ± 0.11	10	0.72 ± 0.14

B MATED BEETLES

Dry weight in mg ± S.E.

Beetle age	n	Non-infected	n	Infected
15 days	10	1.14 ± 0.23	10	1.89 ± 0.49
30 days	9	2.03 ± 0.28	9	1.76 ± 0.26

Fig. 8.2

Uptake of ^{14}C-Leucine and labelled protein by the ovaries of infected and non-infected T. molitor

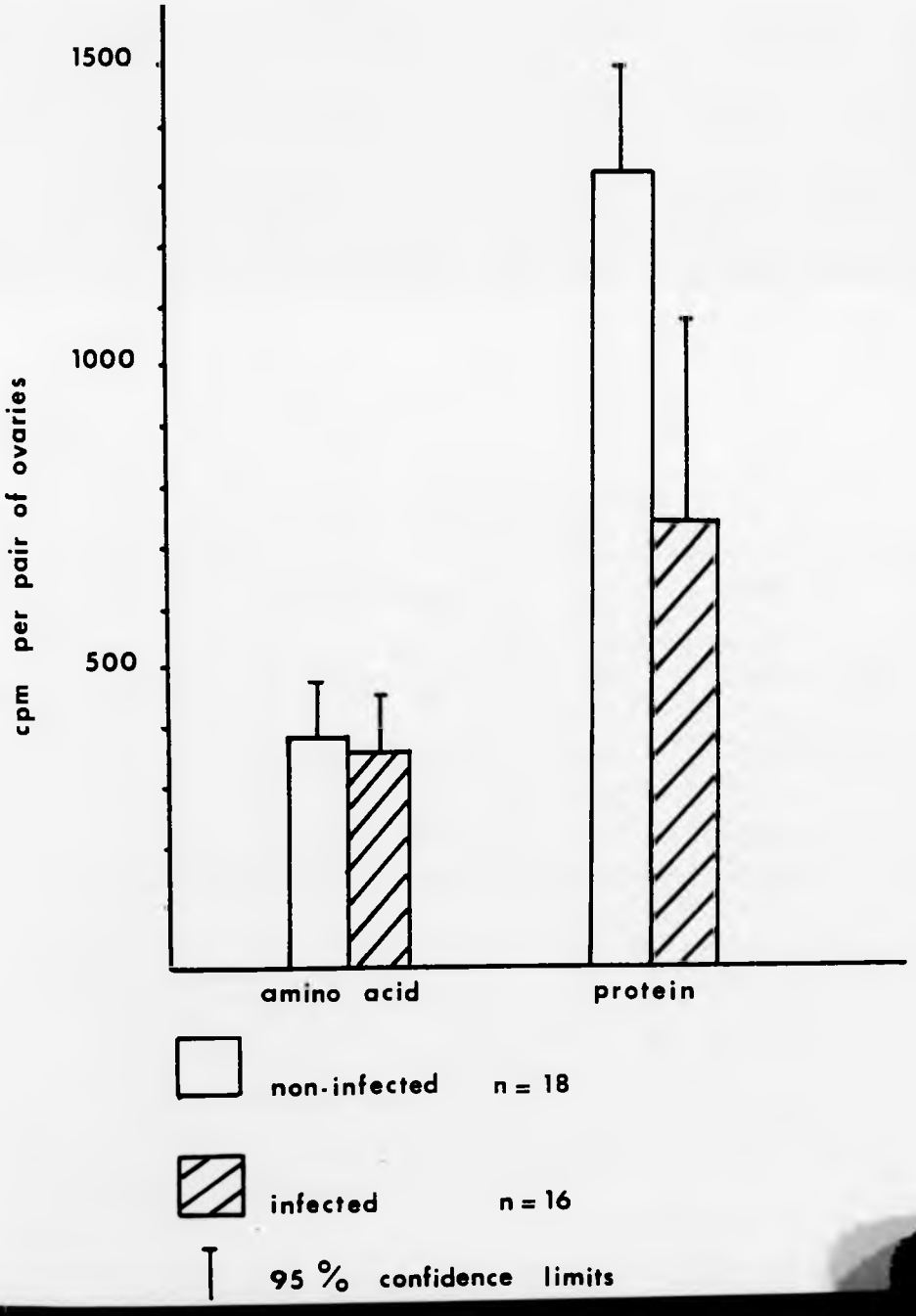


Table 8.2 The incorporation of ^{14}C - into ovaries from T. molitor after a 4h in vivo incubation.

A AMINO ACIDS

Counts per min/mg wet weight ± S.E.

Beetle age	n	Non-infected	n	Infected
9 days	8	90.4 ± 11.8	8	121.1 ± 18.5
12 days	8	69.1 ± 16.2	8	62.2 ± 19.5
15 days	9	181.9 ± 66.3	9	82.2 ± 24.9

B PROTEINS

Counts per min/mg wet weight ± S.E.

Beetle age	n	Non-infected	n	Infected
9 days	8	292.0 ± 29.7	8	332.3 ± 33.8
12 days	8	380.1 ± 107.5	8	230.3 ± 100.9
15 days †	9	519.0 ± 66.9	9	252.1 ± 87.4

† Sig. diff. values, $p < 0.05$

However, by day 15, ovaries from infected beetles contained 51.15% less labelled protein 4h after injection.

A representative densitometric scan of the profile of ovaries from a 15 day-old non-infected female after 4h *in vivo* incubation with ^{14}C-leucine is shown in Fig. 8.4. The two major peaks correspond to the haemolymph vitellogenic proteins 2/3 and 7/8 identified in Ch. 6. The distribution of ^{14}C incorporated into proteins separated by SDS PAGE is shown in Fig. 8.3. The electrophoregram from the corresponding sample in the stained section of the gel demonstrates that the majority of label was detected in the vitellogenin subunits 2/3 and 7/8 (Fig. 8.3) however, most subunits contained some activity.

DISCUSSION

A comparison of dry weights of ovaries from non-infected and infected beetles at all ages examined revealed no significant differences, although ovaries from 15 day-old infected females were shown to contain far less labelled protein after a 4h *in vitro* incubation period than those from non-infected beetles. Further data pertaining to egg production are presented in the following chapter, where a decrease in protein content is demonstrated in eggs from infected beetles. It is possible that, due to the small contribution made by proteins to the total ovary weight, any difference resulting from protein concentration was not detectable using this technique.

The presence within the ovaries of *T. molitor* of radiolabelled proteins after a 4h incubation with ^{14}C-leucine (injected into the haemocoel) can be explained in two ways: the proteins could have been synthesised *in situ* from labelled amino acids; alternativly, proteins synthesised by the fat body and released to circulate in the haemocoel may have been sequestered by vitellogenic ovaries during

Fig. 8.3 An electrophoretic profile of radiolabelled T. molitor ovaries

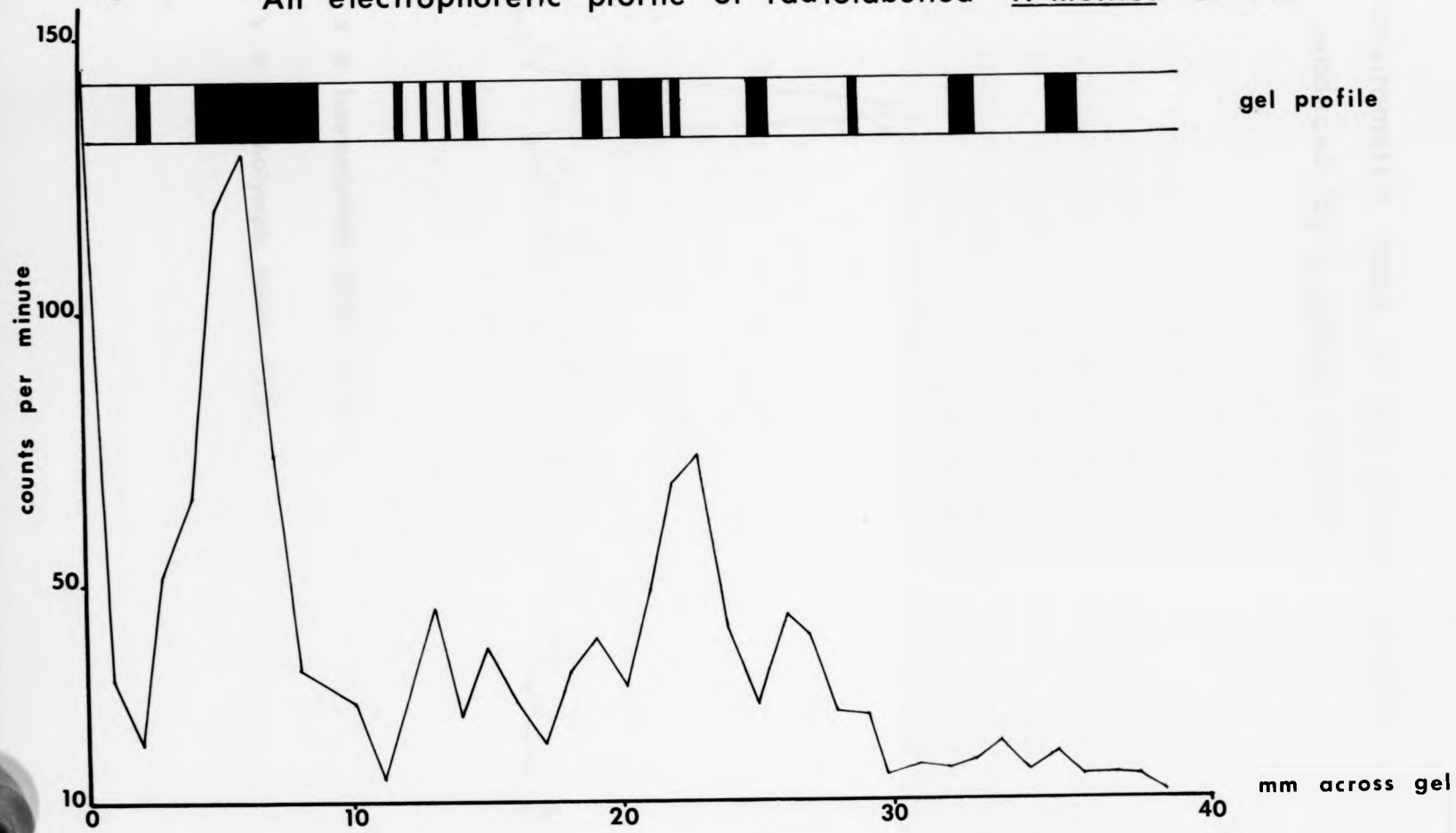
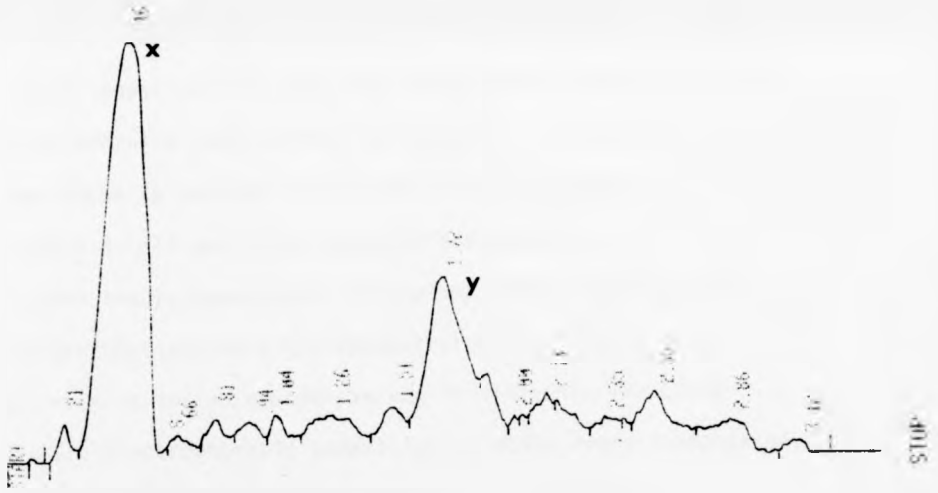


Fig. 8.4

Densitometric trace of the protein profile produced by T. molitor ovaries



x ≡ haemolymph band 2/3

y ≡ haemolymph band 7/8

this incubation period. Ullmann (1973), using autoradiographic techniques, was able to detect the presence of labelled proteins within the ovarian tissue of T. molitor 30 min after in vivo incubation with labelled amino acids. After 1.5 - 6h she demonstrated a pattern of protein incorporation into peripherally formed yolk spheres, no incorporation being detected in chorionated oocytes. The appearance of labelled nurse cells, ooplasm and follicle cells suggested the occurrance of some protein synthesis in situ, although she was unable to demonstrate the passage of labelled protein from trophocytes to oocytes, or to show any follicular contribution to the yolk spheres. She concluded that yolk was sequestered from the haemolymph proteins via the interfollicular spaces. In a recent review, Bradley (1983) summarised the mounting evidence in support of the view that vitellogenic ovaries actively synthesise proteins in a variety of insects. Follicle cell products have been shown to include vitellogenic binding proteins, vitellogenin itself and other unidentified proteins.

In this study, separation of labelled ovarian proteins into their constituent subunits has demonstrated that the majority of the radioactivity is indeed present in the vitellogenic fractions (Fig. 8.2). Electrophoretic separation of whole ovary homogenates, prior to the vitellin purification procedure used in Ch. 6, demonstrated the presence of several minor bands (Fig. 8.3 & 8.4), many with similar Rf values to haemolymph polypeptides (Ch. 6), in which a little radioactivity was also detected. An in vitro culture technique (Ch. 7) has demonstrated that the fat body is capable of the synthesis and release of a considerable quantity of labelled protein during a 4h incubation. It is thus believed that the majority of the radioactivity detected in ovaries incubated in vivo is incorporated within vitellogenic proteins, sequestered from the

haemolymph pool, synthesised and released by the fat body during a 4h period. The origin of the minor labelled protein fractions is uncertain. They too could have been sequestered, for it is known that insect oocytes take up small amounts of haemolymph proteins other than vitellogenins. Alternatively it is possible that synthesis *in situ* from the ovarian pool of labelled amino acids has occurred. The suppression of vitellogenesis in infected insects is well documented and has been discussed in Ch. 1. Gordon, Webster & Hislop (1973), for instance, observed a sessation of vitellogenesis accompanied by resorption of the penultimate oocytes 3 weeks after nematode infection of desert locust. *H. diminuta* metacestodes do not appear to have such a pronounced effect upon *T. molitor* for vitellogenesis was shown to be still occurring in 15 day-old infected beetles, albeit at a much reduced rate.

Evidence presented in this chapter provides an explanation for the elevated vitellogenin concentrations detected in haemolymph from females 12 days or more post-infection. No significant difference in vitellogenin sequestration was observed before this date, and it is proposed that between 9-12 days post-infection, parasitism is directly or indirectly associated with an impairment of the uptake of vitellogenin, thereby causing its build up within the haemolymph. There are many accounts in the literature of a similar increase in haemolymph vitellogenin concentration resultant upon ovariectomy, for instance in a cricket (Cheng & Bradley, 1983), a cockroach (Englemann, 1979) and the Colorado beetle (De Loof & De Wilde, 1970). Chinzei, Chino & Wyatt (1981) made use of the technique of ovariectomy to obtain an accumulation of vitellin in concentrations suitable for purification procedures in *Locusta migratoria* and Borovsky (1981) suggested that the build-up of vitellogenin following ovariectomy of *Aedes* spp. acted as a negative feedback mechanism to

inhibit its synthesis in the fat body.

The importance of the CA in the control of vitellogenesis was first demonstrated by Wigglesworth (1936), and the current understanding of the effects of juvenile hormone upon the ovaries has been summarised in an essay by Davey (1981). Allactomy was shown severely to reduce patency (Pratt & Davey, 1972a), and juvenile hormone applied to vitellogenic ovaries in vitro increases the patency as measured on a subjective scale (the patency index) of intercellular space size, (Davey & Huebner, 1974). Davey (1981), discussing the mode of action of juvenile hormone, observed that the follicle cell response involved a rapid decrease in volume, the removal of fluid probably being linked to a membrane change. Ouabain proved to be a potent inhibitor of juvenile hormone action, and a four-fold increase in Na^+K^+ ATPase activity was demonstrated in isolated follicle cell membranes exposed to the hormone.

The involvement of the CA and its products in ovarian sequestration has also been demonstrated in a locust (Ferenz, Lubzens & Glass, 1981). The use of the compound precocene, to inactivate the CA, has enabled Wilson, Landers & Happ (1983) to suggest that juvenile hormone deficiency resulted in a reduction in vitellogenic oocytes in Drosophila melanogaster ,and Aldrich, Soderlund, Bowers & Feldlaufer (1981) to demonstrate that the uptake of vitellogenin by ovaries slowed down in the absence of this hormone. The CA of T. molitor have been shown to produce juvenile hormone (Lender & Laverdure, 1964a, 1964b) and the relationship between the endocrine system and oocyte development was discussed by Mordue (1965a, b, c). Using a combination of histological techniques, allatectomy, neurosecretory cell cautery and nervi corpris cardiaci sectioning, he found that the neurosecretory cells produce an allotrophic hormone which passed along the nerves to the CA. In vitro incubation with

ecdysone demonstrated that this hormone is essential for oocyte growth and differentiation, different concentrations being needed for each process. The presence of farnesol methyl ester, a juvenile hormone precursor, inhibited growth and differentiation (Laverdure, 1970). However, investigating vitellogenesis *in vivo*, she showed that decapitation at the time of the last larval moult inhibited vitellogenesis, but re-implantation of brain and CA from female, male or larval *T. molitor*, initiated it. Laverdure (1972) also demonstrated that if CA removal was delayed by 48h, oocytes initiated vitellogenesis, but protein uptake later ceased. Implantation studies also led her to suggest that diet stimulates CA activity and that this is under the influence of the brain. Products of the CA were thought to control the synthesis and release of vitellogenins by the fat body.

The effect of juvenile hormone upon the patency of the follicular epithelium in *T. molitor* has not yet been investigated. The use of precocenes for these studies would appear to be impractical as *T. molitor* has been shown to be insensitive to treatment *in vivo*, although some CA activity is lost following *in vitro* incubation (Bowers & Feldlaufer, 1982). The fact that haemolymph vitellin is not a limiting factor in ovarian protein sequestration in infected beetles would suggest that uptake may be inhibited at the follicular epithelium or oolemma, possibly as a result of interference by the parasite with the host endocrine control of this process. This hypothesis will be discussed further in Ch. 11.

Chapter 9

MEASUREMENT OF FECUNDITY IN NON-INFECTED AND INFECTED BEETLES

INTRODUCTION

Ovaries from 15 day-old T. molitor, infected with metacestodes of H. diminuta, have been shown to sequester vitellogenic proteins at a reduced rate when compared with ovaries from non-infected beetles of the same age (Ch. 8). It was thus considered appropriate to determine whether a reduction in yolk uptake is associated with ovulation/oviposition of fewer eggs than normal, or the production of smaller and/or less viable eggs.

Tenebrio molitor exhibits an asynchronous pattern of egg development, much variation existing in both daily laying pattern and overall fecundity. Ullmann (1973) suggested that this reflected the difference in the number of ovarioles per ovary between beetles, and Gerber (1975) confirmed this variation, reporting a mean of 31 ovarioles per beetle (range 27-35), ovaries from the same female usually containing a different number of ovarioles. Schlottman & Bonhag (1956) and Mordue (1965a), however, noted no such variation, and found ovaries to contain only 12 teleotrophic ovarioles. Both Ullmann (1973) and Gerber (1975) calculated that 6 days were required to complete a gonadotrophic cycle, the first eggs being oviposited by virgin females 5 days post-emergence. Following ovulation, many eggs are retained for varying periods in the lateral oviducts (see Fig 8.1). Gerber (1975) recorded a maximum of 30 eggs retained by virgin females on day 10.5, and 21 eggs retained by their mated counterparts. He also found that virgin females laid fewer eggs (1-3 per day from day 6.5-12.5) than mated insects (17-18 per day from day 6.5-12.5) and that mated females initiated oviposition sooner.

Mordue (1965a) was in agreement with this, and demonstrated a greater rate of oocyte production in mated females, oocyte resorption, which began on day 11-12 in virgins, never being observed in mated females. Gerber (1975) suggested that stimuli associated with mating enhanced oviposition whilst accumulation of eggs in the oviducts inhibited oocyte maturation in virgins. Mordue (1965a) also found that crowding stimulated oocyte growth. Eggs are deposited within the food material (Gerber, 1975 and Gerber & Sabourin, 1984) and also at the bottom and sides of the containers.

In view of the information cited above, two methods were used to assess egg production: oviposition was monitored for 30 days post-emergence and the total eggs ovulated calculated by addition of the number of retained oocytes present in the oviducts on day 30. A comparison of the volume and protein content of eggs retained by infected and non-infected females was also made and the viability of eggs laid by single pairs and beetles kept in crowded conditions was determined.

MATERIALS AND METHODS

All insects used for these studies were maintained as described in Ch.2; however, the bran diet of paired beetles and those kept in crowded conditions was supplemented with apple. The spermathecae of paired and crowded females were dissected in 0.9% sodium chloride solution at the end of each experimental period and examined for the presence of sperm. Data from unmated beetles were excluded from the studies of paired beetles.

1. Egg production

A comparison of egg production by mated non-infected and infected females was carried out with beetles maintained under two different regimes: in single pairs (30 pairs) and in crowded conditions of 8 males and 8 females per Petri dish (14 groups). Beetles were paired on emergence, infected as described in Ch.2 or maintained as controls. On day 6, and every alternate day until day 30 post-emergence, beetles were removed from their containers, and the paper lining, the sides of the dish and the bran were examined under a dissecting microscope, the number of eggs laid being noted. The beetles maintained in pairs were replaced in new dishes, and the used Petri dishes and eggs were kept at 26° C, and examined each day for egg hatching. Eggs laid by beetles kept in crowded conditions were removed from the containers and the beetles replaced, additional bran being added when necessary.

On day 30, female beetles were dissected in a solution of 0.9% sodium chloride and the number of oocytes retained in the lateral oviducts noted. Four groups of beetles (2 infected and 2 non-infected) were also maintained in crowded conditions, but with 12-14 females and fewer males per container. Egg laying and egg viability for these very crowded groups was ascertained as above. Virgin females lay fewer eggs; and therefore egg production in this group was assessed entirely by measurement of oocytes retained in the lateral oviducts of 9, 15 and 30 day-old infected and non-infected females. This parameter was also determined for 9, 15 and 30 day-old mated beetles.

2. Oocyte volume

Ovaries were dissected from 9, 15, and 30 day-old virgin non-infected and infected beetles and 9, 15, and 30 day-old crowded mated females and placed in a few drops of 0.9% sodium chloride solution on a coverslip. The lateral oviducts were slit and retained oocytes teased out. Measurements of length and width of the yolk content of each oocyte were made using a Vickers microscope (30x magnification) fitted with a micrometer eyepiece. Volume calculations were made using the formula for a regular elipsoid:

$$\frac{3}{4}\pi\frac{L.W^2}{2.4}$$

3. Total soluble protein content of oocytes

The protein content of oocytes retained in the lateral oviduct of 15 day-old infected and non-infected beetles was determined as follows. Oocytes were removed from the lateral oviducts and 10 selected at random and pooled in 0.5 ml of the buffer used for vitellin preparation (Ch. 6). The eggs were broken open with a mounted needle and homogenised on ice. The homogenate was centrifuged for 10 min at 4° C (9000 g)and the pellet washed in a further 0.5 ml buffer and centrifuged as before. The supernatants were pooled, added to 2 ml ice-cold TCA and left for 1h at 4°C. The protein was precipitated by centrifugation for 15 min at 4° C (1000 g) and the precipitate resuspended in a further 2 ml TCA and centrifuged as before. The resultant precipitate was dissolved in 4.8 ml 0.5 N NaOH and the protein content of 1.6 ml aliquots determined by the Lowry method described in Ch.4.

RESULTS AND DISCUSSION

The majority of females in this study commenced egg laying 6-7 days post-emergence; however, the time of first oviposition ranged from day 5-16. By comparison, Gerber (1975), maintaining T. molitor at 28° C, found that mated females began oviposition on the 4th day post-emergence. This discrepancy in the initiation of oviposition could be the result of a maintenance temperature difference or due to the 2 day starvation period used in this work.

The opaque, white, fragile eggs were laid singly or in clusters and stuck to the sides of the Petri dish and on the paper lining it, or were dispersed throughout the food. The scattered eggs were covered in adhering bran particles and faecal pellets which necessitated the tedious hand sorting of bran with fine forceps to locate them. The mean egg length, determined as 1.61 mm, was smaller than that of 1.75-1.8 mm reported by Cotton & St. George (1929) but comparable to stage 5 of Gerber's (1975) oocyte maturation classification, which consisted of chorionated oocytes, 1.4-1.6 mm in length.

The mean number of eggs produced in 30 days by single pair non-infected beetles was 128.5. Mordue (1964a), assessing oocyte production in terms of the combined measurement of size of developing terminal oocytes and the number of full-term eggs produced, found crowded mated females to have a significantly greater rate of oocyte production than single pair mated females. The role of pheromones in the acceleration of egg production in T. molitor was discussed by Happ, Schroeder & Wang (1970), and Happ & Wheeler (1969) concluded that male pheromones promote rapid egg laying. Gerber (1975) however, in a comparison of oocyte production in mated and virgin females, dismissed the idea that pheromones were involved in

promoting egg laying. He argued that all beetles were kept in the same room, and therefore exposed to similar concentrations of pheromones, and yet mated females produced more eggs. He also pointed out that, for most of the period of his study (the first 6 days post-emergence), the beetles were not producing pheromones due to sexual immaturity. He suggested that stimuli associated with copulation and/or insemination enhanced egg deposition in T. molitor, rather than exposure to male pheromones. The role that pheromones play in the stimulation of oocyte maturation or egg deposition in this study is unknown. All beetles were kept in the same incubator but in closed Petri dishes; therefore a gradient of pheromones could develop around single pair or crowded beetles. It may be pertinant to note that, in overcrowded conditions, beetles were usually returned to the original bran after each egg search. However, on one occasion fresh bran was provided to 3 of the groups and in each case egg laying fell dramatically (see Fig. 9.1), whereas no such reduction occurred in the group that was maintained in the original bran throughout the experiment. To eliminate the variable that pheromone impregnated food may introduce, all beetle groups used in the crowded condition experiment were maintained in the same bran throughout the 30 day period. Data concerning the number of eggs laid, eggs retained and the total ovulated by single pair, crowded and very crowded beetles are summarised in Table 9.1. In contrast with the findings discussed above (Mordue, 1964a), crowded conditions were not found to enhance egg production or laying. However, beetles kept in very crowded conditions (11-14 female beetles per Petri dish) ovulated fewer eggs than those kept in single pairs or crowded conditions, and, whilst fewer eggs were laid, more were retained within the lateral oviducts. The decline in egg-laying towards the end of this experiment affected two infected groups and only one

Table 9.1

Total eggs ovulated by 30 day old beetles.

SINGLE PAIRS

Eggs per beetle	n	Non-infected	n	Infected
	30		30	
Eggs laid		113.1 ± 11.3		99.1 ± 13.1
Eggs retained		14.8 ± 1.8		16.1 ± 1.8
Total ovulated		127.9		115.2

CROWDED CONDITIONS

Eggs per beetle	n	Non-infected	n	Infected
	55		55	
Eggs laid		111.8		110.6
Eggs retained		16.7 ± 1.3		13.5 ± 1.17
Total ovulated		128.5		124.1

VERY CROWDED CONDITIONS

Eggs per beetle	n	Non-infected	n	Infected
	24		21	
Eggs laid		61.8		39.3
Eggs retained		23.1		18.9
Total ovulated		84.9		58.2

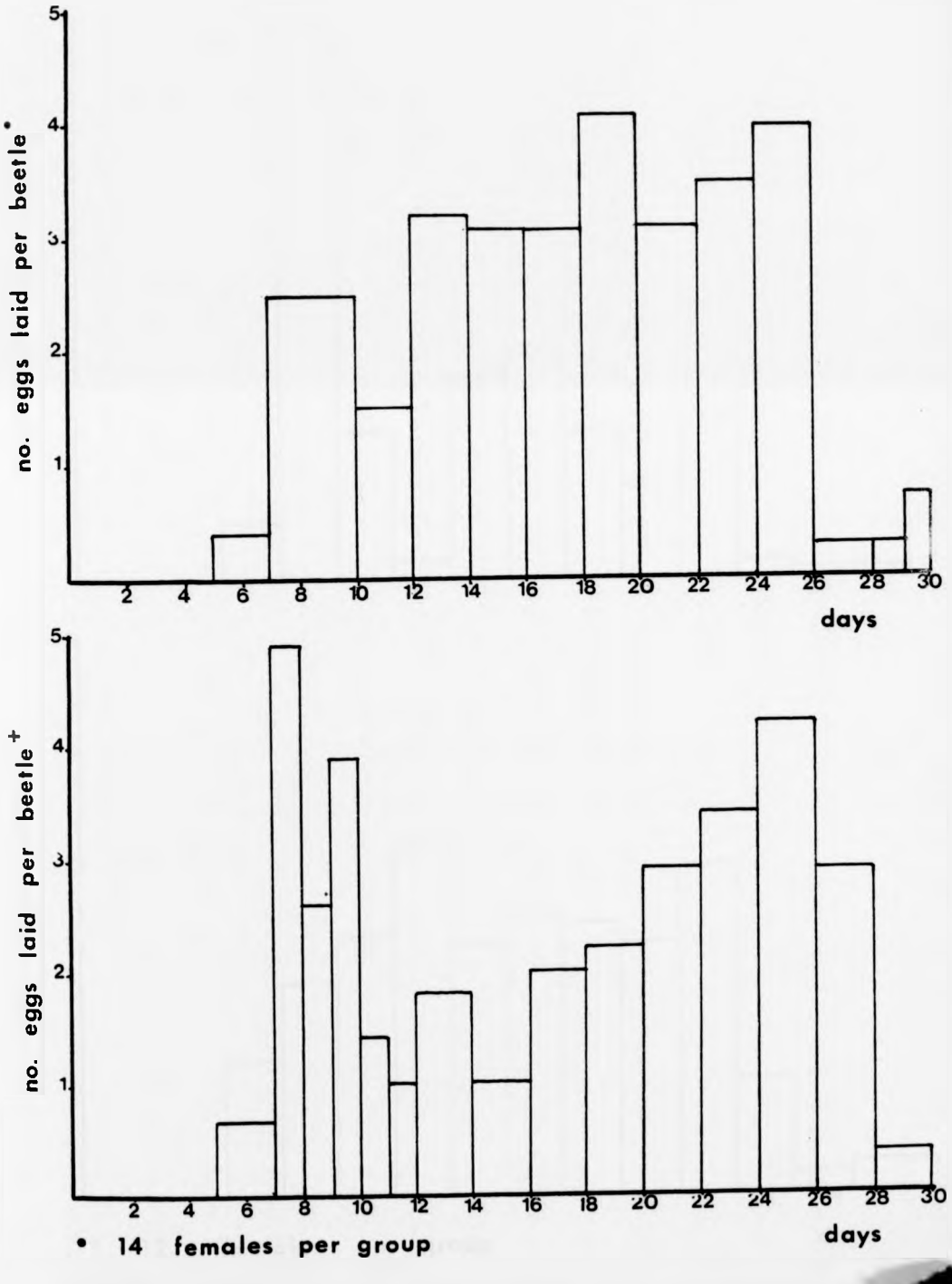
non-infected group; this did not, however, produce the overall difference between non-infected and infected beetles. Calculations based on egg-laying for the first 23 days, the period prior to bran removal in all groups, also showed a reduction in egg laying, from 44.84 eggs per non-infected beetle to 30.16 eggs per infected beetle. Data concerning egg-laying per beetle, expressed on a 24h basis, revealed that beetles kept in crowded conditions laid more eggs throughout the experiment than those in very crowded conditions.

The fecundity of beetles infected with metacestodes of H. diminuta was unaffected by infection when hosts were kept in single pairs or crowded conditions. However, the number of eggs layed per beetle in 30 days declined from 84.93 to 58.23 in infected beetles kept in very crowded conditions. Although the explanation for this difference is unknown, it is possible that an additional factor resulting from overcrowding exists in the latter group, which causes reduced fecundity in non-infected beetles and a further reduction in infected ones. All beetles received adequate food supply, and moisture was added to the diet by the provision of fresh apple; thus no element of food competition is thought to have existed in very crowded conditions. An increase in oxygen consumption has been detected in infected Tribolium spp.(Soltice, Arai & Scheinberg, 1971) that was attributed to increased respiration on the part of the host tissue rather than parasite metabolism. Oxygen has been shown to play only a minor role in the physiology of adult H. diminuta (Fioravanti & Saz, 1980), and Schiller (1965) was able to cultivate metacestodes to maturity in vitro in an anaerobic environment. It is thus conceivable that, in very crowded conditions, available oxygen became a limiting factor contributing to reduced fecundity in infected beetles.

Observation of egg laying in the 4 groups of very crowded beetles indicated a cyclical pattern of egg laying over the first 30 days post-emergence, 3 peaks occurring within this period (see Fig. 9.1) Ullmann (1973), examining oviposition in T. molitor over a 4 month period, was unable to detect any such pattern and described egg laying as asynchronous. In order to investigate this further, 14 groups of beetles were maintained in the crowded conditions described above, and egg laying monitored on a 48h basis. Mortality was low, no deaths occurring after the first 10 days post-emergence. Dead males were replaced by younger ones, females, however, were not replaced because of the need to standardise ages. Results represented in Fig. 9.2 are therefore expressed on the basis of eggs laid per beetle per two days and are a mean of 55 non-infected and 55 infected females. Three peaks in egg laying were again observed. The first and third, on day 8-10 and 26-28 respectively were common to both conditions. The second peak, however, which occurred on days 16-18 in non-infected beetles, was apparently delayed in infected beetles, occurring on days 20-22. Two-way analysis of variance using an Avona table: mixed design was performed on a GEC 4082 computer (Keele biology statistics library) to test the significance of the relationship between: egg laying/time in non-infected beetles, total egg laying in non-infected/infected females and egg laying pattern in non-infected/infected females. Egg laying was found to vary significantly with time ($p < 0.0042$), ovulation appearing to occur in a cyclic fashion for the first 30 days. Peaks in egg laying detected in this work are separated by periods of 8 and 10 days respectively, thus suggesting a longer gonadotrophic cycle than the 6 day cycle discussed above (Ullmann, 1973 and Gerber, 1975). There was a significant difference in this pattern between non-infected and infected beetles ($p<0.03$), the presence of metacestodes causing a

Fig. 9.1 a

T. molitor egg laying pattern in non-infected beetles

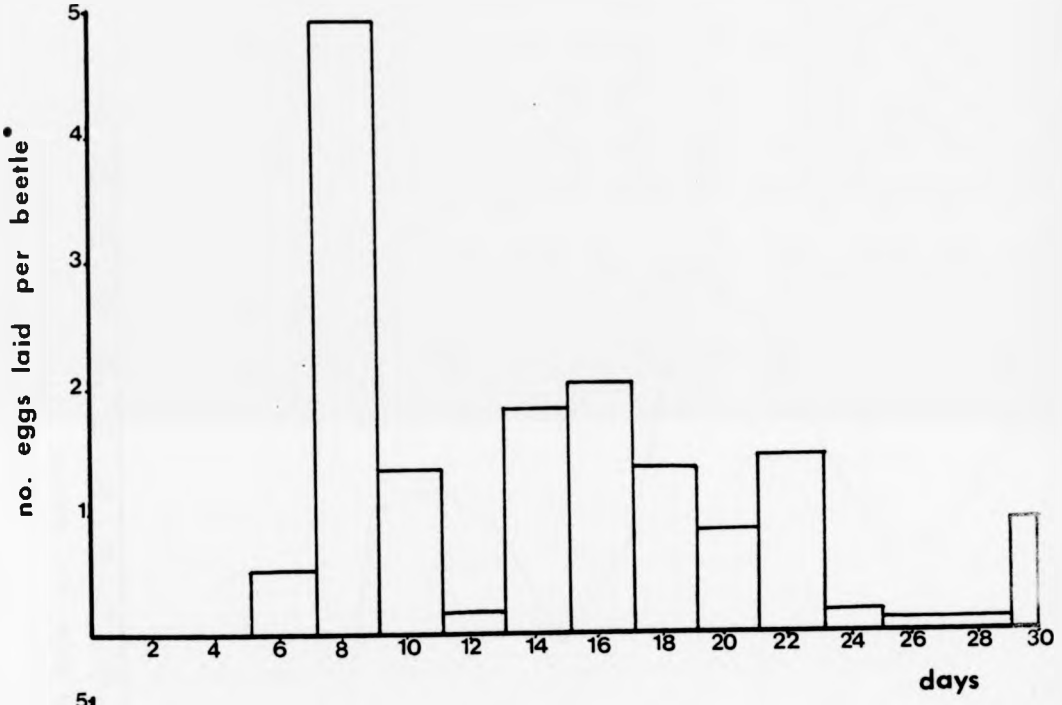


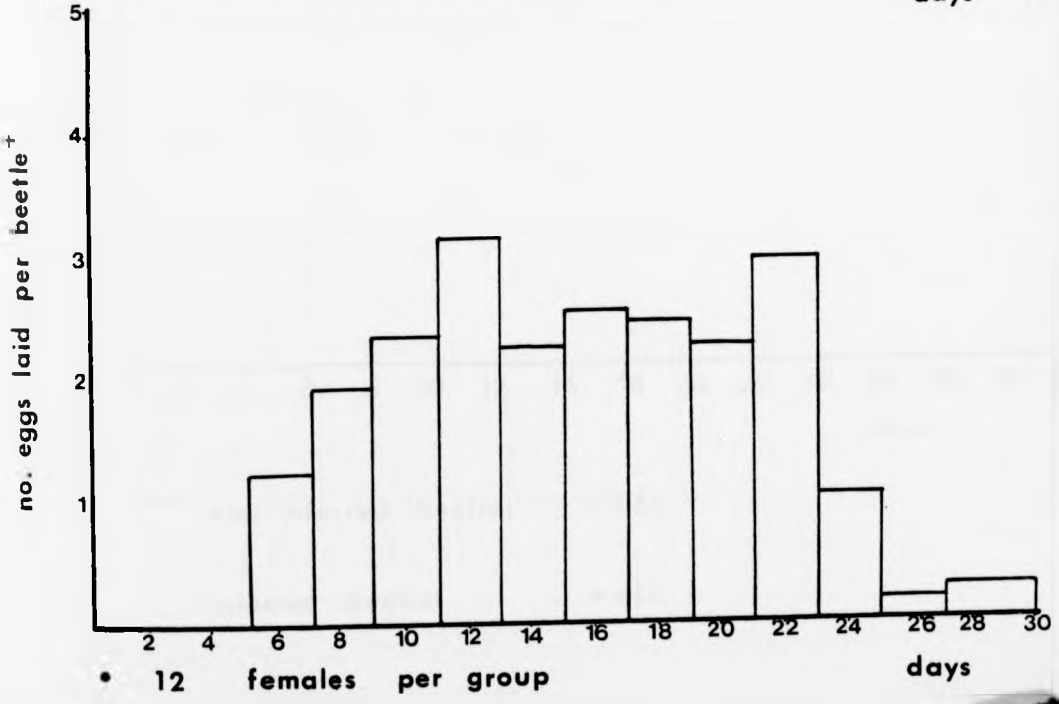
• 14 females per group

+ 10 females per group

Fig. 9.1b

T. molitor egg laying pattern in infected beetles



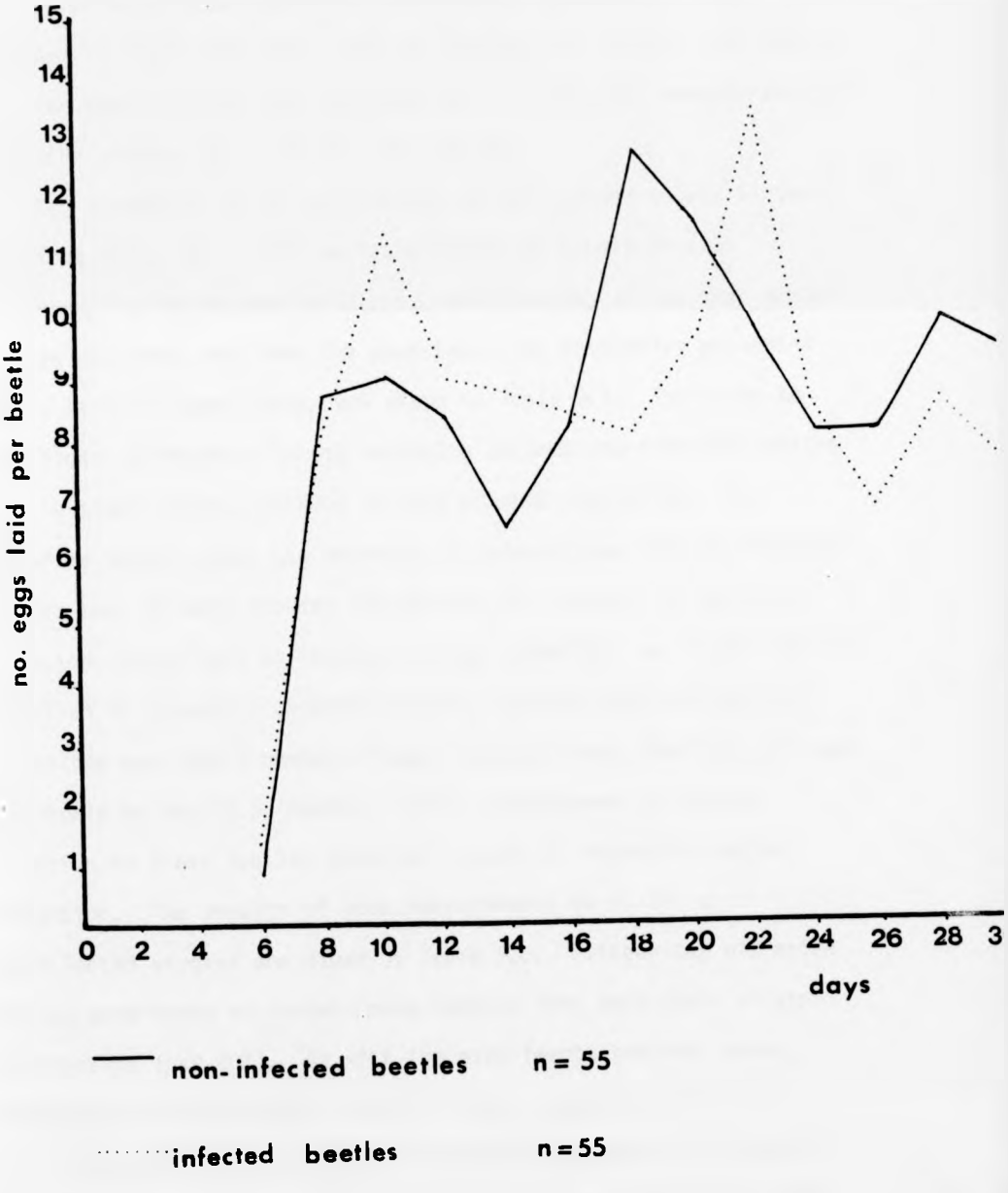


• 12 females per group

+ 9 females per group

Fig. 9.2

T. molitor egg laying pattern for 30 days post emergence



delay in the second peak of egg laying. However, no difference was found in the total number of eggs laid by normal and infected beetles, thus confirming the results expressed in Table 9.1. It is relevant to note that the period during which the oviposition rate differs between non-infected and infected beetles corresponds with the period when an elevation in vitellogenic proteins is first detected in haemolymph from infected beetles (Ch. 4 & 6), and also to the time when ovaries from infected beetles have been demonstrated to sequester protein at a reduced rate (Ch.8).

Egg production in T. molitor cannot be assessed solely in terms of oviposition, due to the variable number of mature oocytes retained in the lateral oviducts. Calculations of the mean number of eggs retained, and thus the mean total egg production per mated female in all 3 conditions, are given in Table 9.1. There was no significant difference in egg retention between non-infected beetles kept in single pairs, crowded or very crowded conditions. It therefore appears that the decrease in oviposition, seen in infected beetles kept in very crowded conditions, is a result of decreased ovulation rather than an increase in egg retention, as is the overall reduction in fecundity observed in very crowded infected beetles. Due to the very small number of eggs laid by virgin beetles, (18 eggs per female by day 12.5 (Gerber, 1975)), measurement of oocyte retention by these beetles provided a means of assessing oocyte production. The results of such measurements on 9, 15 non-infected virgins are given in Table 9.2. Fifteen-day old mated females were found to contain more oocytes than both their virgin counterparts ($p<0.002$). As with the experiments outlined above, parasitization was found to have no effect upon egg retention.

The results of egg volume measurements are shown in Table 9.3. No significant difference in the yolk content was detected in eggs

Table 9.2

Eggs retained in the lateral oviduct of virgin *T. molitor*.

Beetle age	Eggs retained per beetle			
	n	Non-infected	n	Infected
9 day	10	18.1 ± 1.9	10	13.3 ± 1.8
15 day	10	17.5 ± 2.0	10	14.3 ± 2.4

Table 9.3

Yolk volume of ovulated eggs of T. molitor.

	Yolk volume per egg in mm^3 ± S.E.			
Female beetle	n	Non-infected	n	Infected
9 day virgin	82	0.520 ± 0.010	82	0.518 ± 0.010
†15 day virgin	77	0.370 ± 0.007	77	0.410 ± 0.014
15 day mated	100	0.515 ± 0.009	100	0.501 ± 0.008
*†30 day virgin	75	0.206 ± 0.019	39	0.110 ± 0.023
30 day mated	92	0.532 ± 0.010	92	0.510 ± 0.014

* non-infected and infected significantly different $p < 0.01$

† significantly different $p < 0.001$

Table 9.4

Viability of eggs laid by 30 day old beetles.

	% Hatch rate			
	n	Non-infected	n	Infected
30 day old	1445	60%	770	52%

from non-infected and infected virgin beetles 9, $\overset{\circ\circ}{\lambda}$15, despite the fact that sequestration of protein by the ovaries of 15 day-old virgins is known to be significantly depressed in infected beetles. As stated previously, virgin T. molitor lay few eggs, and most of those ovulated during the first gonadotrophic cycle are retained within the oviducts. It has been suggested (Gerber, 1967 & 1975) that the retention of mature oocytes inhibits further oocyte production. The majority of eggs retained within 15 and 30 day-old females are thus likely to be products of the first gonadotrophic cycle and, as such, underwent vitellogenesis at a time when infection had no effect upon yolk uptake (ie. prior to day 15). Retained oocytes were undergoing oocyte resorption in both non-infected and infected beetles, a significant reduction in volume occurring between day 15 and 30. Mordue (1965a) reported that resorption of oocytes in virgin females begins 11-12 days post-ecdysis. No such resorption occurred in oocytes retained by mated females in which continuous ovulation and oviposition presumably results in a much shorter retention time. The volume of yolk in oocytes from 30 day infected virgin females was significantly smaller than their non-infected counterparts. These data are based on oocytes from 5 beetles only and a larger sample should be examined before any conclusions are drawn concerning egg-size reduction at a time when, in mated 9, no difference in vitellogenesis has been detected. Eggs from infected 15 and 30 day-old females taken from a larger sample of beetles, showed a small reduction in volume. It would thus appear that metacestodes of H. diminuta have no effect upon the volume of eggs produced by their host, nor upon the rate at which resorption proceeds in virgin females. This is in contrast with the 20% reduction in size of oocytes produced in the beetle Dendroctonus pseudotsugae infected by Contortylenchus reversus (Thong & Webster,

1975a).

A difference was, however, detected in the total TCA insoluble protein content of eggs from non-infected and infected 15 day-old virgins, which fell from 15.26µg per egg to 13.6µg in infected females ($p < 0.05$). These data are from 10 determinations, each made on 10 pooled eggs, and, assuming a mean of 15 retained oocytes per beetle, represents an 11% reduction in total egg protein output per beetle, assuming a constant rate of oviposition. The majority of the oocytes retained within the oviducts of 15 day-old virgin beetles would have undergone vitellogenesis and been ovulated prior to 9 days post-emergence. Thus, unless reabsorption is occurring at a faster rate in infected beetles, these findings indicate that vitellogenesis has been affected by the metacestodes 6 days or less post-infection. This is in contrast to the findings described in the previous chapter which demonstrated that a significant decrease in vitellogenin uptake by ovaries from infected beetles was detected at 15 but not at 12 days post-emergence. It would also indicate that the parasites are affecting egg protein content at an earlier period than detectable changes in the haemolymph soluble protein occurred. The discrepancy between these results and those pertaining to egg volume could be due to the small proportion of total egg volume occupied by vitellins.

The viability of eggs from non-infected beetles was low, 63% hatch for single pair beetles and 60% hatch for very crowded beetles. This is in contrast to the 90% hatch rate reported by Keymer (1980) for Tribolium confusum and may be due to: a large proportion of the eggs being unfertilized, a high mortality rate for developing eggs or unsuitable incubation conditions. Comparison of the percentage hatch of eggs from infected and non-infected beetles revealed a decrease in hatch rate with parasitization in both the single pair (7.4%) and very crowded conditions (Table 9.3). The effect of infection upon

egg viability may be the result of: the reduction in protein content of eggs from infected beetles described above, a reduction in some other factor resultant upon infection or to unknown factors affecting sperm viability.

The effect of metacestodes of H. diminuta upon the various parameters of fecundity described above has demonstrated that the parasite influences the population dynamics of the beetle. Although ovulation and oviposition do not cease with infection, and indeed, over a 30 day period no reduction in egg laying occurred, both the delay in the second oviposition cycle, and the decrease in percentage hatch rate, would retard the growth of infected populations to some extent. The effects are, however, slight by comparison with the examples described in Ch.1. These effects not unique to insects but also occur amongst both invertebrate and vertebrate hosts. For example, Ligula intestinalis infections in cyprinid fish cause a suppression of gonadotrophin production from the pituitary gland resulting in an inhibition of gametogenesis (Arme, Bridges & Hoole, 1983). Von Brand (1979), summarising data concerning the parasitic castration of molluscs by juvenile trematodes, showed that the degree of host egg-production depends upon the parasite species present. He dismissed both mechanical damage and indirect effects of parasite-induced starvation as mechanisms of snail castration in the majority of cases, and discussed the view that hormonal influences may be involved, although no supporting evidence exists. However, Looker & Etges (1979) related a decrease in fecundity in Schistosoma mansoni- infected Biomphalaria glabrata to nutrient depletion by the parasite. The pathology of spathebothriidean infections in gammarids has been reviewed by Freeman (1983), who described parasitic castration resulting from Diplocotyle olriiki infections.

Reports of the influence of cyclophyllidean metacestodes upon

host reproduction are confined to those of; Dowell & Jones (1966), who produced evidence to support the hypothesis that infection of T. confusum by H. microstoma reduces host reproductive potential, Evans (1983) who reported a reduction in fecundity of the ostracod Herpetocypris reptans infected with Hymenolepis tenerrima and Keymer (1980 & 1981) investigating the infection of T. confusum with H. diminuta. By means of repeated exposures to infective eggs, Keymer (1980) was able to increase parasite burden, which reached a plateau with single infections, in a linear manner. She found that fecundity decreased as mean parasite burden increased, although no difference was detected in the percentage hatch of eggs from infected and non-infected beetles. A long-term study, conducted over a period of 60 weeks (Keymer, 1981), revealed a 60% depression in the population levels of T. confusum infected with H. diminuta. A comprehensive study involving collection of data from beetles with various levels of infection would need to be conducted to investigate this fully. However, from data obtained in this study it seems unlikely that H. diminuta exerts such profound effects upon T. molitor population dynamics.

Chapter 10

HAEMOLYMPH FREE AMINO ACIDS IN INFECTED AND NON-INFECTED T.MOLITOR

INTRODUCTION

The majority of endopterygote insects contain high concentrations of haemolymph free amino acids (HFAA) (Duchateau & Florkin 1958). Sutcliffe (1963), in his discussion of the chemical composition of insect haemolymph, described four basic types characterized in terms of the major components that contributed to total osmotic pressure. He suggested that in the Exopterygota, classed as types I or II, sodium and chloride were major constituents. On the other hand, chloride only accounted for a minor part and sodium formed the major osmotically active constituent of types III & IV. Tenebrio molitor, unlike the majority of the Coleoptera, was placed in type IV along with the Lepidoptera and Hymenoptera. Here a major contribution to the osmotic pressure of haemolymph is made by free amino acids, with concentrations ranging from 83-166 mM. In addition to their role in osmoregulation, HFAA contribute to the buffering capacity of insect blood and are utilized in metabolism.

Many insects are reported to achieve some degree of homeostatic control of HFAA, although the mechanisms are poorly understood. Collett (1976) described the storage of amino acids in Drosophila and Calliphora as intracellular peptides of 5 amino acids or less. These provide a source of amino acids with which to maintain osmotic stability as the rate of protein turnover varies. The assimilation and degradation of these peptides is regulated by the action of haemolymph peptidases, which are inhibited by high concentrations of HFAA. Bosquet (1977) suggested a similar regulatory mechanism in the

silkworm Philosamia cynthia where, with the exception of ornithine, concentrations of amino acids remained stable during starvation whereas amounts of all peptides declined. Woodring & Blakeney (1980), investigating the role of HFAA in osmoregulation of cricket blood during starvation, implicated the specific amino acids proline, glycine and tyrosine in the maintenance of total amino acid concentrations. They proposed that either blood peptides or haemocytes could be a source of these specific amino acids in periods of stress. The findings of Evans & Crossley (1974) also provided evidence of a homeostatic function for haemocytes. They showed that the dicarboxylic amino acids, glutamate and aspartate, were localized in the haemocyte fraction of Calliphora haemolymph and suggested a protective role for these cells in maintaining plasma glutamate at low concentrations.

Not all insects, however, can regulate their HFAA concentrations. For instance, Horie & Watanabe (1983b) found that in Bombyx mori larvae, HFAA manifested characteristic patterns according to the type of dietary protein provided. In addition to the dietary "stress" noted above, the presence of parasites also influences HFAA. Literature concerning the effects of haemocoel parasites on host HFAA is extensive and diverse, some of this having been discussed in Ch.1. In general, effects of unicellular parasites upon their hosts appear to be more acute than those caused by metazoans. Thus, Thong & Webster (1975a) found no significant change in HFAA concentrations of Douglas Fir beetles infected with the nematode Contortylenchus reversus, and Lackie (1972) could detect no differences in HFAA concentrations in Periplaneta americana infected with Moniliformis dubius.

Prior to the work described in this chapter, (see Hurd & Arme, 1983; Hurd & Arme, 1984a) there have been no reports of the effect of

H. diminuta metacestodes on the HFAA of its intermediate host. The present study reports changes in HFAA of infected and non-infected T. molitor at different ages and different stages of infection.

MATERIALS AND METHODS

Beetles collected from the stock colony (Tenebrio molitor: Rice University Strain) were used for all HFAA determinations. Details of rearing, sexing and infection have been described previously (Ch.2). Haemolymph samples from beetles 9, 12, 15, & 30 days post- emergence were collected and stored at -20°C for a maximum period of 2 weeks. Infected beetles aged 15 days were dissected after haemolymph collection to determine the intensity of infection. Prior to analysis, haemolymph was deproteinized. The plug of haemocytes was removed from the capillary tube and the volume of haemolymph noted. It was necessary to use between 2.5 µl and 4 µl of haemolymph for each analysis because many of the amino acids could not be detected in smaller samples. Haemolymph was then added to 3 ml of ice- cold 3% sulphosalicylic acid in 0.01M HCl, centrifuged at 4°C for 15 min at 25000 g and the supernatant stored at -20°C.

Analysis was carried out on a six-sample, discrete dual-flow column analyser (Model JLC 6AH). Basic amino acids were separated on a 15 cm column using sodium eluting buffers pH 4.56 and 5.90, and acid and neutral amino acids were separated on a 50 cm column using lithium eluting buffers pH 2.78, 3.15 and 3.94 (The composition of these buffers is given in Appendix 9). Amino acid concentrations were determined by a ninhydrin photometric method and the resulting peaks integrated. The use of standard amino acid mixtures (Pierce and Warriner) enabled amino acid peaks to be identified and quantified by comparison with retention times and peak areas of the

standards. Results were analysed using a GEC computer (the programme listing is given in Appendix 10) .

RESULTS

A major part of the analysis of HFAA was undertaken on 15 day-old Tenebrio molitor. However a number of additional observations have been made on beetles aged 9, 12, and 30 days. These have indicated that age-related changes in HFAA do occur in the adult insect (see Tables 10.1 and 10.2). Total concentrations range from 34-94 mM, with a peak occuring 15 days post-emergence. Sixteen HFAA have been detected in beetles of all ages studied, together with methionine which was only present in trace amounts. Alanine and citrulline were not completely resolved as separate peaks and have been included as a composite value. In addition three unknown peaks, possibly peptides, were observed; these were eluted first from the acid-neutral column, appearing before threonine and serine.

Although there are insufficient data from 9, 12 and 30 day old beetles to warrant a statistical analysis, certain trends have been observed. For example, the reduction in proline concentration, described below in 15 day-old female beetles, also occurs in parasitized females 12 days post- emergence, in which there is a 31% reduction. At 9 and 30 days, however, the concentration of this amino acid is apparently unaltered by parasitization. In contrast , in 15 day-old male insects there is no significant difference in the proline concentration of non-infected and infected hosts, although lower values were obtained in the latter at 9, 12 and 30 days post-emergence. The lowering of glycine concentrations, observed in infected male beetles of all ages, does not occur in females. Arginine and serine are found in reduced concentrations in 12 day-old

Table 10.1 and 10.2 legend

* For 9 and 12 day beetles all data are derived from 2 separate determinations from pooled samples.

2 beetles contributed to the pool in each case.

For 15 day beetles all data derived from 7 separate determinations of samples from individual beetles.

For 30 day beetles all data are derived from 2 separate determinations from pooled samples.

3-8 beetles contributed to the pool in each case.

N = non-parasitized

P = parasitized

Table 10.1 Amino acids in female Tenebrio molitor (mM)

Beetle age	9 DAYS		12 DAYS		15 DAYS ± S.E.		30 DAYS	
*	N	P	N	P	N	P	N	P
ORN	0.25	0.38	tr	0.62	0.25 ± 0.09	0.28 ± 0.04	0.30	0.24
LYS	2.60	2.83	3.97	6.42	3.10 ± 0.42	3.82 ± 0.37	3.13	3.12
HIS	5.98	5.45	4.56	4.85	4.52 ± 0.50	4.21 ± 0.32	4.23	3.29
ARG	1.39	1.70	3.06	1.57	1.07 ± 0.20	1.41 ± 0.26	3.70	3.29
THR	0.75	0.84	0.86	0.81	0.84 ± 0.11	1.23 ± 0.20	0.83	0.65
SER	1.10	1.21	1.29	0.77	1.10 ± 0.12	1.32 ± 0.21	1.26	1.17
GLU	0.52	0.44	0.31	0.43	1.51 ± 0.56	1.54 ± 0.51	1.38	1.05
GLN	2.30	2.65	4.77	4.43	2.76 ± 0.81	3.05 ± 0.71	2.49	1.64
PRO	7.89	7.48	31.66	21.76	55.89 ± 10.2	38.63 ± 2.60	15.39	17.65
GLY	1.26	1.43	1.55	1.00	2.09 ± 0.45	2.26 ± 0.39	1.09	0.90
ALA/CIT	2.09	2.70	4.63	5.24	3.60 ± 0.63	2.34 ± 0.32	2.31	2.80
VAL	3.11	2.90	3.47	3.09	3.28 ± 0.71	2.71 ± 0.63	2.86	2.39
ISO	0.36	0.70	1.79	2.83	1.03 ± 0.10	1.36 ± 0.26	1.19	1.49
LEU	1.68	1.93	1.83	2.95	1.19 ± 0.19	1.49 ± 0.21	2.00	2.35
TYR	2.11	2.28	2.16	2.03	2.70 ± 0.21	2.22 ± 0.24	1.68	0.96
PHE	0.86	0.64	0.54	0.55	0.59 ± 0.06	0.42 ± 0.06	0.50	0.33
TOTALS	34.25	35.56	66.45	59.35	85.51	68.30	44.33	43.36

Table 10.2 Amino acids in male Tenebrio molitor (mM)

Beetle age	9 DAYS		12 DAYS		15 DAYS ± S.E.		30 DAYS	
*	N	P	N	P	N	P	N	P
ORN	0.24	0.65	tr	tr	0.25 ± 0.07	0.32 ± 0.07	0.17	0.26
LYS	3.17	2.92	4.91	4.42	3.81 ± 0.80	3.45 ± 0.75	2.86	4.82
HIS	4.23	5.29	5.30	4.61	4.5 ± 0.38	3.82 ± 0.37	3.29	4.07
ARG	1.14	1.25	1.14	1.65	2.20 ± 0.75	0.88 ± 0.11	3.69	5.41
THR	0.56	0.79	0.60	0.63	0.78 ± 0.12	0.94 ± 0.17	0.58	0.57
SER	0.71	1.18	1.04	0.89	1.28 ± 0.17	1.43 ± 0.23	1.06	0.76
GLU	0.73	0.39	0.25	0.36	1.23 ± 0.45	1.50 ± 0.31	1.42	2.11
GLN	1.56	2.65	2.46	2.60	2.15 ± 0.59	2.63 ± 0.81	1.96	1.61
PRO	10.95	6.79	23.42	21.74	58.1 ± 12.97	54.1 ± 11.16	21.53	10.69
GLY	1.07	1.66	1.28	1.40	1.86 ± 0.32	2.74 ± 0.54	0.62	0.89
ALA/CIT	1.76	2.88	5.60	3.23	3.08 ± 0.59	2.86 ± 0.68	1.96	2.55
VAL	3.18	4.25	3.63	3.96	4.06 ± 0.61	4.01 ± 0.57	2.66	2.92
ISO	0.55	1.14	1.68	2.60	1.26 ± 0.27	1.02 ± 0.12	1.03	1.20
LEU	1.53	2.06	1.30	2.33	1.26 ± 0.26	1.25 ± 0.24	1.70	1.66
TYR	2.33	3.07	1.87	1.08	2.34 ± 0.32	2.64 ± 0.48	1.16	1.15
PHE	0.53	0.91	0.55	0.66	0.33 ± 0.10	0.51 ± 0.16	0.48	0.35
TOTALS	34.24	37.23	55.03	52.16	94.18	84.10	45.70	40.66

parasitized females, a reversal of the condition found in 15 day-old insects. In infected males, arginine is elevated on day 12, but is significantly lower than non-infected controls on day 15.

Although variations in the concentrations of a number of HFAA occurred in 9, 12 and 30 day-old beetles, as described above, the majority of samples and the most detailed analysis of changes in individual HFAA concentrations associated with parasitism, involved insects aged 15 days (Tables 10.1 and 10.2). This age was selected because studies described in previous chapters had shown that 15 day-old female beetles, harbouring 12 day-old metacestodes, exhibited an unusual pathophysiological feature; the total protein content of their haemolymph was elevated relative to that of non-parasitized controls.

No significant difference in individual HFAA concentrations has been detected between non-infected male and female beetles. However, following infection certain changes are observed. These are most marked in parasitized females in which nine amino acids are significantly affected: the concentration of isoleucine, leucine, arginine, serine and threonine is raised whereas that of tyrosine, phenylanaline, proline and alanine/citrulline is lowered, relative to non-infected controls (Table 10.3). In parasitized males, only four amino acids are affected: the concentration of threonine and glycine is raised and that of histidine and arginine is lowered ($p<0.05$ for all values).

Dissection of 15 day-old infected beetles revealed intensities of infection ranging from 10-244 metacestodes per insect. A product-moment correlation has been performed to determine whether a relationship existed between the number of metacestodes present and the concentration of those amino acids which were altered by the presence of the parasites. No such correlation was found for any of

Table 10.3 "p" values obtained from the Student t test giving a significant difference between groups tested.

Amino acid	Non-infected female/male	Female non-infected/infected	Male non-infected/infected
ORN	-	-	-
LYS	-	-	-
HIS	-	-	p<0.01 D
ARG	-	p<0.02 E	p<0.02 D
THR	-	p<0.001 E	p<0.05 E
SER	-	p<0.05 E	-
GLU	-	-	-
GLN	-	-	-
PRO	-	p<0.001 D	-
GLY	-	-	p<0.01 E
ALN/CIT	-	p<0.001 D	-
VAL	-	-	-
ISO	-	p<0.01 E	-
LEU	-	p<0.02 E	-
TYR	-	p<0.02 D	-
PHE	-	p<0.01 D	-

D - amino acid concentration depressed in infected beetle

E - amino acid concentration elevated in infected beetle

the ten amino acids tested, namely: proline, threonine, alanine/ citrulline, isoleucine, phenylalanine, leucine, tyrosine, arginine and histidine.

DISCUSSION

The results of this study support the view (Sutcliffe, 1963) that T. molitor haemolymph has a high aminoacidaemia. Average totals, calculated for the seven 15 day-old non-infected female and male beetles examined, are 87.49 mM and 87.31 mM respectively. These results are in accord with observations made on various members of the Insecta, for example, Calliphora vaccina, 54.3 mM (Evans and Crossley, 1974) and Aedes aegypti 40-60 mM (Edwards, 1982).They indicate the significant contribution made by amino acids to haemolymph osmolarity. Measurements of the osmolarity of pooled haemolymph from 15 day-old T. molitor, using an osmometer (Wescor Inc. 5100 series) gave an average reading of 500 mOsM, HFAA therefore making a contribution in the order of 17%. The osmolarity measured here is similar to figures reported for other insects, for instance the blowfly Lucilia sericata, 415-430 mOsM (Irving, Osborn & Wilson, 1979) and the diving beetle Dytiscus marginatus, 401 mOsM (Sutcliffe, 1962).

Total HFAA concentrations were found to vary with age. Data in this study range from 34-94 mM, with a peak occuring 15 days post-emergence. Much of the age-related variation in total HFAA concentration is due to changes in the value of a single amino acid viz. proline. In male beetles aged nine days, proline comprises 32% of the total and the corresponding data at 12, 15 and 30 days are 43%, 62% and 47% respectively. If proline values are subtracted from the totals then little variation in HFAA pool size is observed.

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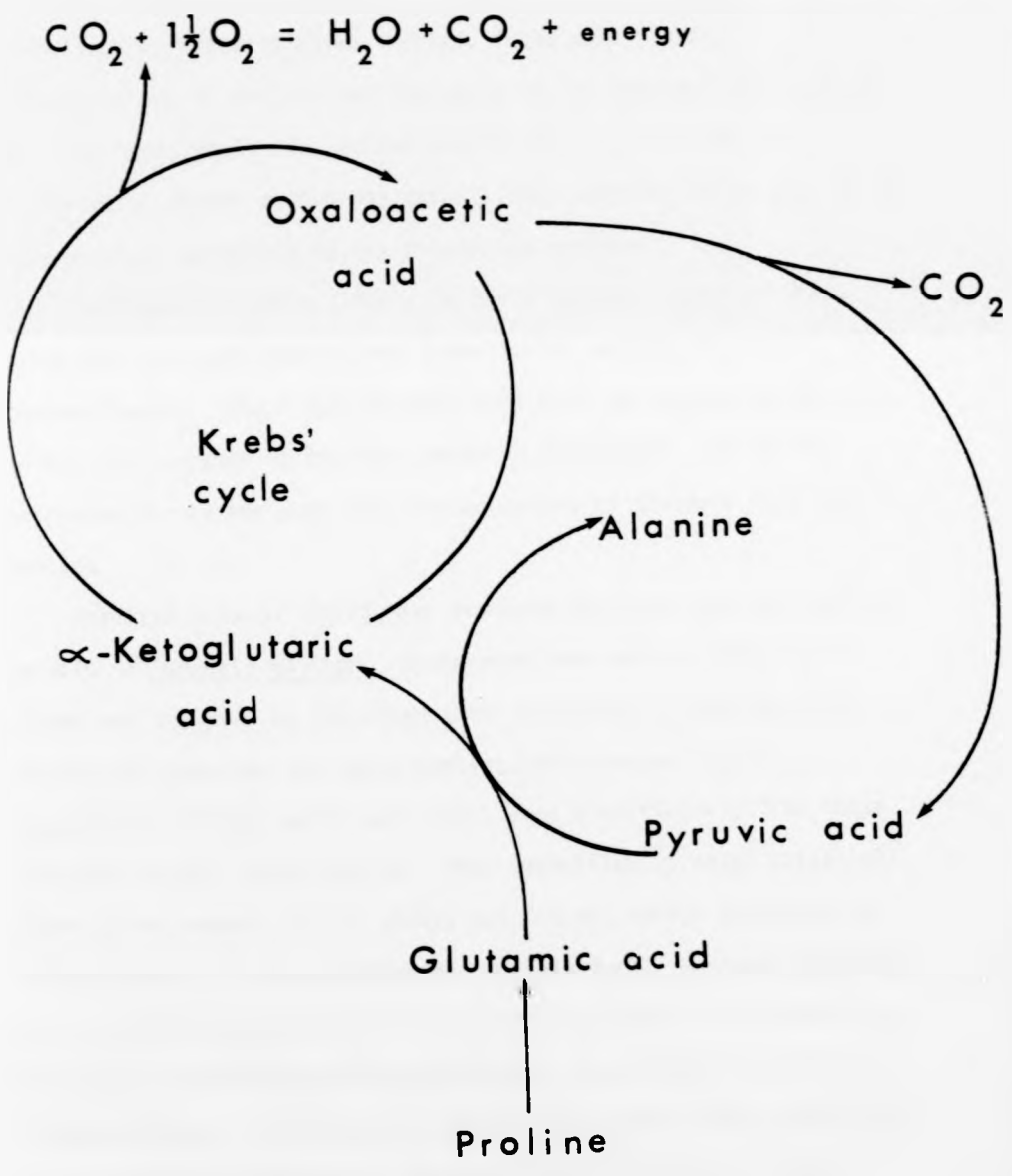
Haemolymph rich in proline is a common feature of insects. Barrett & Friend (1975) found proline to be the predominant amino acid in the haemolymph of Rhodnius prolixus where it constitutes 38.4 - 59.2 % of the total free amino acids detected. Bursell (1963) considered this amino acid to be a readily mobilizable energy reserve for flight in the tse-tse fly (Fig.10.1), where it is also the major HFAA. He related the occurrence of this pathway to the carbohydrate-deficient blood diet of Glossina morsitans. Because haemolymph glucose and trehalose concentrations are low, the oxidation of carbohydrate as a source of energy for flight is precluded, and this role has apparently been assumed by proline. An important function of proline in flight muscle metabolism has also been described in several beetles. Notably, DeKort and co-workers have investigated aspects of proline metabolism in the Colorado beetle, Leptinotarsus decemlineata. Age-related changes in the concentrations of proline (DeKort & Kramer, 1976) follow a pattern similar to that found for T. molitor, with an increase during the first days of imago life. Glutamate was found to play a central role in proline metabolism but, unlike the tse-tse fly, alanine did not, at this time, appear to be an end-product of metabolism. However, Weeda, Koopmanschap, De Kort & Beenakkers (1980), analysing proline synthesis in the fat body of L. decemlineata, established that alanine was the only nitrogenous end-product of proline oxidation. They found that fatty acids were the main source of acetyl-CoA for proline synthesis and suggested that the capacity to synthesise proline might be under neuroendocrine control.

The high proline concentrations of Tenebrio haemolymph cannot be explained in terms of a diet low in carbohydrates, as the beetles feed on cereals. The requirement of proline as a rapidly mobilizable energy reserve for flight muscles similarly seems unlikely, for

Fig. 10.1

A hypothetical interpretation of energy mobilization in the tse-tse fly



flight plays very little part in the locomotion of this insect. As Rapport, Yang & Injeyan (1980) observed,the advantage for many insects of the abundance of this particular amino acid has not been adequately explained. However, a role for proline in osmoregulation has been suggested by several authors. For example, Chamberlin & Phillips (1982), investigating the osmoregulation of locust haemolymph by the Malpighian tubules, found much higher concentrations of proline and glutamate in the tubule-fluid compared with the haemolymph. The active secretion of proline and its reabsorption in the rectum was established, and the use of proline as a respiratory substrate by the rectum was proposed.

Duchateau & Florkin (1958), in their general survey of HFAA, point out that each species had a particular pattern of aminoacidaemia. Their data demonstrated that the higher values found, for instance in the more advanced Coleoptera, are mainly accounted for by the very high concentration of glutamic acid and proline.

Bradfish & Punzo (1977) have produced the only previous analysis of HFAA in Tenebrio molitor. Haemolymph from various life-cycle stages was analysed by two-dimensional thin-layer chromatography. The results obtained for adult beetles, with respect to the composition of total amino acid pool, vary significantly from those obtained in this investigation. They identified 15 amino acids, all found to be present in this study, but did not detect ornithine or serine, found in low concentrations in this work. A steady decrease in total HFAA concentrations with maturation from larva to adult was noted but, with the exception of proline, the amino acid pattern changed little. During the larval and early pupal stages this amino acid was found, together with glutamic acid and glycine, to be a major haemolymph component. In the adult, however, proline made a

relatively minor contribution to the total HFAA concentration. The age of the adult beetles used by Bradfish & Punzo (1977) was not given. It is thus difficult to compare these results with the present study as it has been shown here that proline concentrations vary with the age of the adult.

A further factor that may influence proline concentration is the physiological state of the insect (see above). In the present study, some attempt has been made to control this variable by keeping all experimental beetles under identical housing conditions and by always sampling haemolymph at between 9 and 10 am.

The difficulties in accurate determination of glutamine and glutamic acid in the dual-column automatic amino acid analyser has been discussed by Pye, Stonier & McGale (1978). The concentrations of glutamine detected in this investigation are within the values that give a linear relationship between peak areas and concentration. They found, however, that there was a considerable (up to 35%) loss of glutamine during storage in the analyser sampler unit prior to analysis. The concentrations of glutamine and glutamate given in tables 10.1 & 10.2 may not therefore be accurate determinations of their concentration in haemolymph.

Lack of sufficient data from 9, 12 and 30, day-old beetles precludes the drawing of any firm conclusions concerning trends in both age and sex-related differences due to the presence of parasites. Data from 15 day-old beetles however, has shown that the presence of H. diminuta metacestodes in the haemocoel is associated with a number of effects upon HFAA composition, particularly in females.

Comparisons of average total HFAA concentrations for 15 day-old female non-infected and infected beetles (87.49 mM and 73.52 mM respectively) and 15 day-old male non-infected and infected beetles

(87.31 mM and 92.12 mM respectively) reveal no significant differences associated with sex or parasitization. However, differences do exist between the concentrations of some of the individual amino acids in non-infected and infected beetles. Thus the contribution that the HFAA make to the haemolymph osmolarity shows little variation although the relative composition of constituent amino acids has been affected by the presence of metacestodes of H. diminuta.

It is axiomatic that the rapid process of growth and development of the metacestode will depend upon nutrients, including amino acids, being removed from the haemolymph of the host. It has been shown that cysticercoids possess a number of discrete membrane transport sites for amino acids and that leucine, phenylalanine, alanine and proline are incorporated into protein in vivo (Jeffs & Arme 1982, 1983, 1984, 1985a & 1985b). Thus, if the host cannot compensate for this depletion by increased food intake, then the effects described above could be due to competition for nutrient reserves between the symbionts, a condition that resembles the effects of stress due to starvation.

Other possible mechanisms whereby the parasitism could effect changes in individual HFAA concentrations include competition by the metacestodes for other dietary components, for example pyrixidine (see Horie & Watanabe, 1983a), or increased use of some HFAA for glycogenesis to compensate for possible removal of haemolymph carbohydrates by the parasite. The rapid transformation of alanine into trehalose in T. molitor has been demonstrated by Gordoux, Lequellec, Moreau & Dutrieu (1983). This incorporation rate was enhanced by extracts of corpora cardiaca, indicating that glycogenesis is under hormonal control (see Ch. 11). Weeda (1981) described an increase in proline synthesis and a decrease in alanine

production by the fat body of L. decemlineata following injection of corpora cardiaca and corpora allata extracts.

Alterations in the concentrations of some amino acids described above could thus be brought about by the presence of the parasite, which apparently acts in a dose-independent manner. The fact that the changes in HFAA associated with parasitism are more marked in 15 day-old females than males suggests that either the homeostatic mechanisms of the latter are less susceptible to the effects of cysticercoids, or that factors other than nutrient removal are involved. The results of a comparison of haemolymph proteins described in previous chapters would suggest that the latter is the more likely alternative.

Chapter 11

DISCUSSION

The definition of parasitism is fraught with difficulty, some authors adopting narrowly defined criteria, others attempting a broader view. Examples of the former include emphasis on the nutritional dependence of parasites upon host, but this is clearly only one of the many possible factors involved. Thus Read (1970) discussed the relationship between parasitism and disease and concluded that "many so-called parasites have never been shown to cause overt disease." Sprent (1963) developed the view that a parasite must be recognised immunologically as non-self by its host; however, many parasites remain undetectable, residing in immunologically privileged sites or employing molecular mimicry as a disguise. The ecological aspects of parasitism were considered by Crofton (1971) who defined parasitism in quantitative terms, infection being a form of over-dispersal, the degree of infection affecting the host population. His views have been developed in numerous papers by May & Anderson (see for example Anderson, 1982). Indeed, the usefulness of such narrowly defined terms is recognised as being limited in attempts to encompass the wide spectrum found in heterospecific associations, the degree of parasite-dependence on the host often changing both during its life cycle and with the specific host utilized. Smyth (1979) maintained that the term parasitism should be regarded as relative, and that a variety of associations exist within a range of metabolic dependence, from free-living to 100% parasitic.

Most investigations into parasite metabolism have been performed *in vitro*. Whilst such studies are clearly of value, it must be remembered that the intimate nature of the association between the

symbionts often results in fundamental changes on the part of both parasite and host that can only be revealed by studies on the intact symbiosis *in vivo*. Parasites have clearly evolved many adaptive features necessary to their mode of life, and it is becoming evident that their hosts too show adaptations associated with parasitism (see for example Davies, Hall, Targett & Murray's (1980) discussion of the "welcome-mat" hypothesis). Thompson (1983a) examined a definition of parasitism in terms of the level of integration of the partners and stated that "In most cases, however, the host, while providing nutrition to the parasite, makes physiological adjustments and/or is metabolically altered."

The findings described in this thesis are mainly concerned with the pathophysiological aspects of the metacestode/beetle relationship. However, they have led to an appreciation of the integral aspects of the association, involving both physiological and ecological factors.

The lack of information concerning the pathophysiology of *H. diminuta* metacestodes in their intermediate host has already been noted (Ch.1). This is not the case, however, for the adult tapeworm. Mettrick (1980) has reviewed the work of his group and others on this tapeworm/rat relationship over a number of years, and the pathophysiology of this symbiosis has been summarised recently by Arme, Bridges & Hoole (1983). Infection results in the alteration of several parameters in the gut including: gut micro-organisms, pH, oxidation-reduction potentials, oxygen tension and gut nutrient concentrations and gradients. Arme *et al.* (1983) pointed out the paradox that exists when data concerning the pathogenicity of the adult are examined for, despite the occurrence of the effects listed above, "parasitized animals grow normally and show few, if any, signs of clinical disease". They concluded that "The fact that those

changes result in few gross effects in the rat may be related to a long evolutionary relationship between host and parasite and/or to a compensatory effect of alternative physiological mechanisms, brought into play in parasitized animals."

The results presented in this thesis expose a pronounced pathophysiology associated with the metacestode-invertebrate host relationship, affecting both oogenesis in the female beetle, and amino acid metabolism in both sexes. Thus, analysis of haemolymph protein concentration in infected beetles revealed that only female beetles 12 days or more post-infection were affected. The protein elevation associated with parasitism was confined to the female-specific vitellogenins and concentrations did not continue to rise over a period of 15-30 days post-emergence, after the initial increase. The presence within the insect haemocoel of H. diminuta metacestodes of 12 days or older, was associated with a decrease in fat body vitellogenin secretion and also a decrease in its sequestration by the ovaries. It is possible that the 43.5% difference in protein incorporation detected in ovaries from infected beetles was sufficient to account for the elevated concentrations of vitellogenin measured in the haemolymph, despite its apparent lower rate of synthesis. The possible effect that this reduction in protein sequestration had upon beetle fecundity was discussed in Chapter 9. Egg protein content and viability were reduced with infection and the second cycle of egg production was delayed, although overall egg production was unaffected during 27 days of parasitization. Comparison of HFAA also revealed differences due to infection, these being more pronounced in female beetles. Total amino acid concentrations were unaltered , thus their overall contribution to maintenance of, for example, osmotic control in the host was unaffected. However, considerable variation existed between

the relative concentration of individual amino acids, some being lowered and some raised. The remainder of this final discussion is devoted to an analysis of the implications of these results in the appreciation of the intricate nature of this parasite-host relationship.

It is evident that, in some associations, host sex is an important factor in a consideration of the overall relationship. This raises the wider question of whether metacestodes are more successful in terms of survival, rate of development or incidence and burden of infection, in one sex than the other. The influence of host sex upon infection has been examined by various authors. Male vertebrate hosts were found to be more susceptible to helminth parasites by: Mankau & Hamilton (1972) investigating rats infected with Trichinella spiralis larvae, Dobson (1961) examining Nematospiroides dubius in mice and Ohbayashi & Sakamoto (1966) examining oral infections of mice with Echinococcus multilocularis. Adult H. diminuta have been maintained in vitro without the presence of sex hormones (Schiller, 1965) and in vivo neither progesterone nor testosterone are required for normal worm growth. However, worms maintained in hosts on vitamin deficient diets are affected by host sex. Thus, Addis (1946), examining worm growth, and Beck (1951 & 1952) observing egg production, found that worms in female rats on deficient-diets were stunted, and produced fewer eggs, than those in deficient-diet males or in normal-diet hosts. Beck (1951) also observed that egg production in worms kept in males on a deficient diet for 3 months also declined, although worm size remained the same. Castration of male hosts also resulted in smaller worms (Addis, 1946) with lower egg production (Beck, 1952), and these effects could be negated by testosterone or progesterone administration. Testosterone, but not progesterone, was found to

raise egg production in worms from female vitamin-deficient rats and normal worms were obtained from pregnant vitamin-deficient animals. Investigations into hymenolepid infections in their invertebrate hosts have, however, yielded different conclusions. A higher incidence of H. diminuta infections in female insects was described for Tribolium confusum and T. castaneum by Mankau (1977) and Mankau, Ragnell, Johnson, McLaughlin & Sampson (1971); however, no such differences were detected in T. brevicornis infection (Mankau, 1977). Parasite burden was also found to be higher in female Tribolium spp. (Mankau, 1977) but no difference was detected by Mankau et al. (1971). Kelly, O'Brian & Katz (1967) reported an age resistance to H. diminuta metacestode infections in female Tribolium and Mankau (1980), comparing a pygmy mutant strain of T. castaneum with normal beetles, concluded that size and weight had no significant effect upon incidence of infection. Parasite development was found to be more rapid in female hosts by Soltice, Arai & Scheinberg (1971), using the timing of scolex invagination as a measure of H. diminuta development in T. confusum and T. castaneum. However, Schom, Norvak & Evans (1981), examining H. citelli infections of T. confusum, found development time to be greater in female hosts, with infected females having a shorter survival time than males. Sex differences affecting the incidence of infection, parasite burden, developmental time and host survival of H. diminuta infection of T. molitor have not, to date, been reported. Differences in host pathophysiology between the sexes observed in this thesis could have an influence upon all the above parameters and other conditions, such as a variation in metabolic rate between the sexes (Soltice, Arai & Scheinberg, 1971), could also be involved.

The concept that associations between organisms involve an element of competition for nutrients was discussed in Ch. 1. It is

pertinent to observe here the similarity that exists between some elements of the pathophysiology of H. diminuta infection and those that occur in insects as a result of starvation. A decrease in vitellogenesis, often accompanied by oocyte resorption, has been noted in many case, for example Schistocerca (Highnam, Hill & Mordue, 1966). Lim & Lee (1981) reported increased oocyte resorption, accompanied by a depletion of haemolymph and fat body reserves, in the grasshopper Oxya japonica. Jutsum, Agarwal & Goldsworthy (1975) also detected depleted haemolymph carbohydrate and protein concentrations in starved O. japonica but, in Locusta migratoria migratoides, only reduced carbohydrate content was detected. Weaver & Pratt (1981) suggested that a hormonal mechanism was involved in the modulation of reproductive activity with food availability in Periplaneta americana. They found that formation of the ootheca was delayed, and ovarian development ceased, after two-weeks starvation, and a similar decline in corpora allata (CA) activity was noted. Laverdure (1972) found that, in T. molitor, starvation interfered with normal vitellogenesis and resulted in oocyte resorption. Corpora allata from starved beetles were found to be less active, and she concluded that diet influenced the CA via products from the neurosecretory cells. This was confirmed by Mordue (1956a,b,c & 1967), who determined that continued production of neurosecretion and CA hormone only occurred in fed insects. A supply of nutrients did not, however, appear to be the required stimulus, for water alone was sufficient to ensure continued oocyte development and mid-gut protease synthesis.

It cannot be stated unequivically that the results described in this thesis are not due to direct competition by the parasite for haemolymph metabolites. However, the finding that the observed responses to infection are not related to parasite burden strongly

indicate that other factors may be involved. It is proposed that infection results in a disturbance of the endocrine system of T. molitor and a survey of the relevant literature concerned with the hormonal control of insect egg production has been undertaken to provide a framework in which to assess this hypothesis.

Reproductive activities in insects are highly diverse and finely tuned to enable egg production and deposition to occur in conditions most appropriate to each species. The hormonal control of these processes has been the subject of intense investigation. However, because a majority of studies have been directed towards a relatively small number of species, generalizations are difficult to make. In the following brief review three main areas are explored. First, observations on the general control of egg production in insects, secondly an examination of the information available specifically for T. molitor and thirdly suggestions concerning the possible mode of interference in the process by metacestodes of H. diminuta.

The role that juvenile hormone (JH) plays in both the synthesis of yolk proteins in the fat body and the initiation and regulation of their uptake by developing oocytes, has been discussed elsewhere. However, direct or indirect control of reproduction clearly involves a series of co-ordinated events controlled not only by JH but also by an array of compounds whose functioning is only beginning to be understood. Regulatory activity is not, however, confined to products of the corpora allata, but has been attributed to secretions from various parts of the nervous system and, indeed, from the ovaries themselves. The production of neuropeptides occurs both in the pars intercerebralis and lateral neurosecretory cells of the brain. The secretions are stored and released from the neurohaemal organs, the corpora cardiaca (CC), after passage along the nervi corporic cardiaci interior (NCCI). or nervi corporic cardiaci

exterior (NCCII). The functions of these peptides have been the subject of several reviews (see Goldsworthy & Mordue, 1974 & Girardie, 1983), and some have been implicated in the control of oogenesis. For example: egg-development neurosecretory hormone (EDNS) has been shown to activate the ovaries of Aedes aegypti to produce ecdysone; follicle-cell trophic hormone (FCTH) performs a similar function in the locust at the end of vitellogenesis; the release of the ovulation hormone, myotropin, in Rhodnius prolixus will be discussed in some detail below, and both allotropic hormone and allostatin have been implicated in the control of corpora allata secretions. The discovery in recent years of the presence of moulting hormones or ecdysteroids in adult insects, particularly females, has led to an examination of their possible role in reproduction and the evidence that ovarian-produced ecdysone acts as a gonadotrophic hormone was summarised by Hagedorn (1980).

A plethora of information has been produced concerning the production, circulation and degradation of gonadotrophins in a variety of insects. Recently, however, attention has also been directed towards determining the means of their regulation. Davey (1983) proposed that the existence of three gonadotrophic hormones has been firmly established. These are: JH, a regulatory neuropeptide (antagonising the action of JH) and the myotropic hormone mentioned above.

The principal gonadotrophic hormone, JH, influences: vitellin synthesis and secretion by the fat body (Wyatt, 1980 & Englemann, 1980); the onset and extent of patency in follicular epithelial cells (Davey, 1981); the follicular cell response to JH (Abu-Hakima & Davey, 1979), and the production of trophocytes (Pratt & Davey, 1972a). Its synthesis by the corpora allata is under inhibitory nervous control by the brain (Davey, 1983), haemolymph JH titres

providing a secondary control *via* a negative feedback mechanism. This latter is likely to be a response only to certain critical concentrations however, for low titres of JH analogue were found to stimulate corpora allata JH synthesis in the viviparous cockroach, *Diploptera punctata* (Stay & Tobe, 1981). In a review of CA regulation, Tobe (1980) pointed out that, due to the lack of storage within the CA, release of JH is not regulatory. He also postulated that some control of haemolymph JH concentrations could occur *via* breakdown of the hormone by JH-specific esterase, now identified in several insects. De Kort & Granger (1981) demonstrated the existence of neurohormones, produced by the median neurosecretory cells, having an inhibitory effect on the CA (allatostatins) and a stimulatory effect (allatotropins), and production of the respective hormones was dependent upon day-length in the Colorado beetle. Evidence for the existence of these neuropeptides in other insects was reviewed by Girardie (1983), who described the location of allotropin production in the A_2-neurosecretory cells of *Locusta migratoria* by means of specific electrical stimulation of the peripheral region of the pars intercerebralis.

Evidence for the presence, in *Rhodnius prolixus*, of an antigonadotrophic or oostatic hormone, which prevents vitellogenesis in follicles of ovaries containing mature eggs, was presented by Adams (1980). The observation that exogenously applied JH is unable to induce patency in these previtellogenic cells has led to the conclusion that the hormone acts by blocking the action of JH at its site of action in the ovaries *viz.* the follicular cells. A peptide of ovarian origin, probably complexed to a protein, has been shown to inhibit patency *in vivo*, and a similar mechanism may exist in *Schistocerca gregaria* (Adams, 1980). Since this latter review was published, Davey & Kuster (1981) have located neurosecretory organs,

rich in antigonadotrophic activity, in tissue adjacent to the ovaries of P. prolixus. Huebner (1983) discussed the possibility that these are stretched when the ovaries are expanded by mature oocytes and Davey (1983) summarised evidence for an inhibitory nervous control of the release of this neurosecretary substance.

Myotropin, the third gonadotrophin to be reviewed by Davey (1983), was found to be produced by 10 neurosecretory cells of the pars intercerebralis of mated R. prolixus. Its concentration in the haemolymph peaked at the time of ovulation/oviposition, and it was found to be responsible for contractile activity in the ovaries and oviduct. A peak in ecdysteroid activity was associated with the release of this myotropin and this peak, together with an input of spermathecal hormone resultant upon mating, was thought to signal that the ovaries contained mature oocytes. Huebner (1983), reviewing the evidence for the involvement of electrophysiological interactions within the ovariole as a means of intraovariole control, expressed the view that JH and antigonadotrophic hormone were modulating a highly integrated system.

The occurrence of a fourth gonadotrophic hormone, ovarian ecdysone, has been discussed by Hagedorn (1980). Synthesised as α-ecdysone in the follicle cells, it is converted to β-ecdysone (20-hydroxyecdysone or ecdysterone), the active form in most insects investigated. Peaks in production at the end of a gonadotrophic cycle have been recorded in, for example, Naupheta cineria (Zhu, Gfeller & Lanzrein, 1983) and it has been suggested that this hormone plays a role in CA inactivation at this time (Friedel, Feyereisen, Mundall & Tobe, 1980, and Zhu et al., 1983). Ruegg (1981) demonstrated the induction of myotropin release in Rhodnius prolixus by ecdysteroids.

The Diptera seem to represent a special case within this

generalized view of insect endocrinology in that ecdysone, and not JH, is the principal gonadotrophin. Ecdysone, produced by the ovaries of Aedes aegypti and converted into 20-hydroxyecdysone, has been shown to stimulate fat body vitellogenin synthesis (Hagedorn, Connor, Fuchs, Sage, Schlaeger & Bohm, 1975). Both the brain and the CA were found to be necessary for growth of the primary follicles, and a neurosecretory cell product initiates ovarian secretion of ecdysone. This dual hormonal control of oogenesis in mosquitoes is well illustrated by the work of Guilvard, De Reggi & Rioux (1984). By means of radio-immunoassay techniques, hormone peaks in Aedes aegypti were measured in vivo and JH and ecdysteroid peaks were both linked to follicular development and vitellogenesis.

As can be seen above, research has been limited to a very few species and Tenebrio molitor has unfortunatly not featured prominently in this area. However, those aspects of endocrine control of egg production which have been researched are summarised below, in the hope that this information, together with extrapolations from the work discussed above, will provide a framework within which to speculate on the action of metacestodes upon the system.

The in vitro incubation of CA from Tenebrio molitor by Weaver, Pratt, Hamnett & Jennings, (1980) demonstrated that only the lower homologue, C_{16}JH, was produced by this gland. The work of Laverdure (1970) in establishing a link between JH and vitellogenin synthesis in the fat body of T. molitor has been discussed in Ch. 7. Mordue (1965c & 1967) concluded that the size of the CA was controlled by both hormonal factors and via the NCCI. Gland size and aspects of the histology of the neurosecretory cells could not, however, be correlated with secretory activity. He found that neurosecretory cells were activated during the imaginal moult, their products being

necessary for mid-gut protease activity, and that they acted synergistically with the CA to control vitellogenesis.

Briers & De Loof (1981 & 1983) have established the presence of ecdysteroids in whole body extracts of T. molitor, using immunoassay techniques. Their second study identified ecdysone, and highly polar metabolites, to be the prominent ecdysterone, and this is also the case in L. decemlineata (Briers Stoppie & De Loof, 1982). An initial ecdysone peak, present in both sexes, was thought to be necessary for the maturation of gonadal tissue (see the study by Szoppa & Happ, 1982 on male accessory glands). Laverdure (1970 & 1975) found that ecdysteroids, added to in vitro ovary cultures, stimulated cell division and follicle cell organisation, the β form being more active than α-ecdysone. In older female beetles, peak height could be correlated with oviposition, suggesting a physiological function for moulting hormone.

However, it must be emphasised that, to date, no measurements have been made of the concentration of these hormones in haemolymph of T. molitor, and their possible roles in either fat body vitellogenin synthesis or in indirect control of vitellogenesis must therefore remain a matter for conjecture.

Many investigations have been made into the relationships between metazoan parasites and insect endocrine systems. For example, Palm (1948) suggested that the lack of a relationship between parasite number and effect upon host ovaries in Bombus infected with the nematode Sphaerularia, was indicative of such an interaction. The pathophysiological effects of both nematode-insect and insect-insect associations have, in some cases, been interpreted in terms of an alteration in host-hormone balance and were briefly discussed in Ch.1. Various effects of Mermis nigrescens upon its locust host have, often with little evidence, thus been attributed.

For example (Gordon & Webster, 1971) described alterations in water balance, and changes in protein metabolism and fat body reserves were observed by (Gordon, Webster, & Mead, 1971). However, Davey & Hominick (1973) suggested that, although direct intervention in the host endocrine system was possible, the pathology may represent a reaction to stress, resulting in release of hormones, and Craig & Webster (1974) found that mermithid inhibition of moulting did not result in a decrease in host ecdysone. However, Gordon, Webster & Hislop (1973) suggested that parasite-induced changes in locust endocrine systems may be responsible for the impairment of vitellogenesis. Mermithid infection of larval blackflies was found to result in an alteration in CA activity and stored neurosecretory material concentration (Condon & Gordon, 1977).

Girardie & Granier (1974), investigating the parasitic castration of the grasshopper Anacridium aegyptium, by larvae of the dipteran Metacemyia calloti, found the activity of the CA to be unaffected. This was confirmed by Girardie & Girardie (1977b); using electrical stimulation of the pars intercerebralis (PI) and ^{35}S-cystine incorporation into the PI and corpora cardiaca, they showed the activity of the median neurosecretory cells to be reduced in parasitized grasshoppers. The haemolymph protein deficiency, seen in infected insects, was attributed to hypoactivity of the median neurosecretory cells, and they proposed that an impairment of protein synthesis resulted in a lack of vitellogenin for sequestration by the eggs (Girardie & Girardie, 1977a).

Recent developments in the techniques for insect hormone assay have enabled more detailed investigation of the complexity of parasite/host-hormone interactions to be undertaken, and examples from parasitoids and Protozoa are given below. To illustrate the effect that parasitoids can have upon host metamorphosis, mediated

via the endocrine system, the pathophysiology of the infection of Manduca sexta by the parasitoid Apanteles congregatus will be described. The parasite suppressed metamorphosis and it has been shown by Reckage & Riddiford (1982a) that infected larvae have higher JH titres than non-infected counterparts. Reduced JH-specific esterase activity was detected and it was concluded that the parasite suppressed catabolism of JH bound to haemolymph carrier-protein, possibly due to interference with synthesis of the latter in the fat body. They concluded that reduced JH-esterase titres may have resulted in high JH concentrations which would in turn delay metamorphosis. However, increased JH production in vitro was also detected (Kramer & Law, 1980) in parasitized insects. Jones, Jones & Bhaskaran (1981) suggested that low haemolymph trehalose concentrations in starved larvae may have stimulated allatotropin secretions by the brain and this led Reckage & Riddiford (1982a) to suggest that reduced trehalose in infected larvae could similarly be a contributary factor to increased CA activity, although the absence of allatoinhibin neurosecretory factor was also postulated. Because they found host ecdysteroid concentration, at the time of parasite emergence, to be similar to that in unparasitized larvae at the wandering stage, Reckage & Riddiford (1982a) concluded that lack of host behavioural change prior to moulting was due to an elevation in JH rather than lack of moulting hormone.

Protozoa also exhibit interesting interactions with their insect hosts. Thus, the neogregarine Farniocystis triboli inhabits the fat bodies of Tribolium castaneum and, in an advanced stage of infection, destroys the organ. Moulting is then delayed, or severely affected, with larval/pupal or pupal/adult intermediates resulting. Rabindra, Balasubramanian & Jayaraj (1981) suggested that this parasite induces a hormonal imbalance resulting in JH accumulation, possibly due to

lack of JH-degrading enzyme, epoxy hydrolase, normally present in fat body tissue. Inhibition of moulting was also reported by Listov (1977), who studied the effects of the sporozoans Nosema whitei and Adelina triboli upon T. molitor. He concluded that most hormonal changes were due to fat body destruction.

There have been no reports to date of the effect of metacestodes upon the endocrine systems of their insect hosts; however, some are known to intereact with those of vertebrates in a remarkable way. Thus, for example, Spirometra mansonoides appears to produce a growth-hormone like substance that causes enhanced growth of infected rodents and Ligula intestinalis infections of certain cyprinid fish may result in a suppression of gonadotrophin production from the pituitary gland (see Arme et al., 1983) None of the findings presented in this thesis provide direct evidence for parasite interference with the endocrine system of T. molitor. However, as discussed above, aspects of reproduction and also amino acid metabolism that are affected by the parasite, (see Ch. 10) are under hormonal control. A parasite-induced decrease in concentrations of JH would result in the lowered rate of both vitellogenesis and vitellogenin synthesis and secretion observed in infected beetles, and it is felt that this may be the most likely explanation for the recorded observations. For the remainder of this discussion the suggestion that there exists in infected beetles a potent CA inhibitor, acting directly on the glands or via the neurosecretory cells, will be used as a working hypothesis.

The initial decision to use virgin beetles for this study was made in an attempt to reduce the number of variables within the model system, and to minimize the additional demands upon infected females resulting from a high rate of egg production. A retrospective appreciation of some of the pathophysiological effects involved in

this symbiosis has led to the conclusion that further analysis, using mated females, would help to elucidate some of the paradoxes discussed in Ch. 9. It is possible that oocytes retained within virgin females in some way suppress vitellogenesis *via* decreased CA activity, and this feed-back system could act synergistically with the stimulus produced by the parasite. In contrast, in mated beetles, a more active endocrine system may be less susceptible to influence by the parasite, significantly elevated protein concentrations being detected much later than in infected virgins.

Several findings in this study led to the conclusion that the timing of both the possible stimulus presented by the parasite and the response made by the host, are important factors to be considered. All data confirmed the initial observation that, at least with regard to vitellogenesis, pathophysiological effects are not evident during the first 12 days of infection. Two explanations for this present themselves; first, the metacestodes produce some dose-independent factor at, or after, a particular stage in their development or secondly, that the necessary stimulus is present throughout the infection but, due to host physiological factors, does not take effect immediately. The finding that ingestion of heat-inactivated *H. diminuta* eggs resulted in a haemolymph protein elevation, similar to that produced by developing metacestodes, leads to the conclusion that the first of these explanations is probably incorrect. An investigation of egg shell, membranes or oncosphere to determine whether factors were present that were heat stable, insusceptible to digestive enzymes and capable of being absorbed through the insect gut, would be a valuable extension to the present study.

The fact that initiation of JH production in *T. molitor* occurs before the starvation and infection regime adopted in this study has

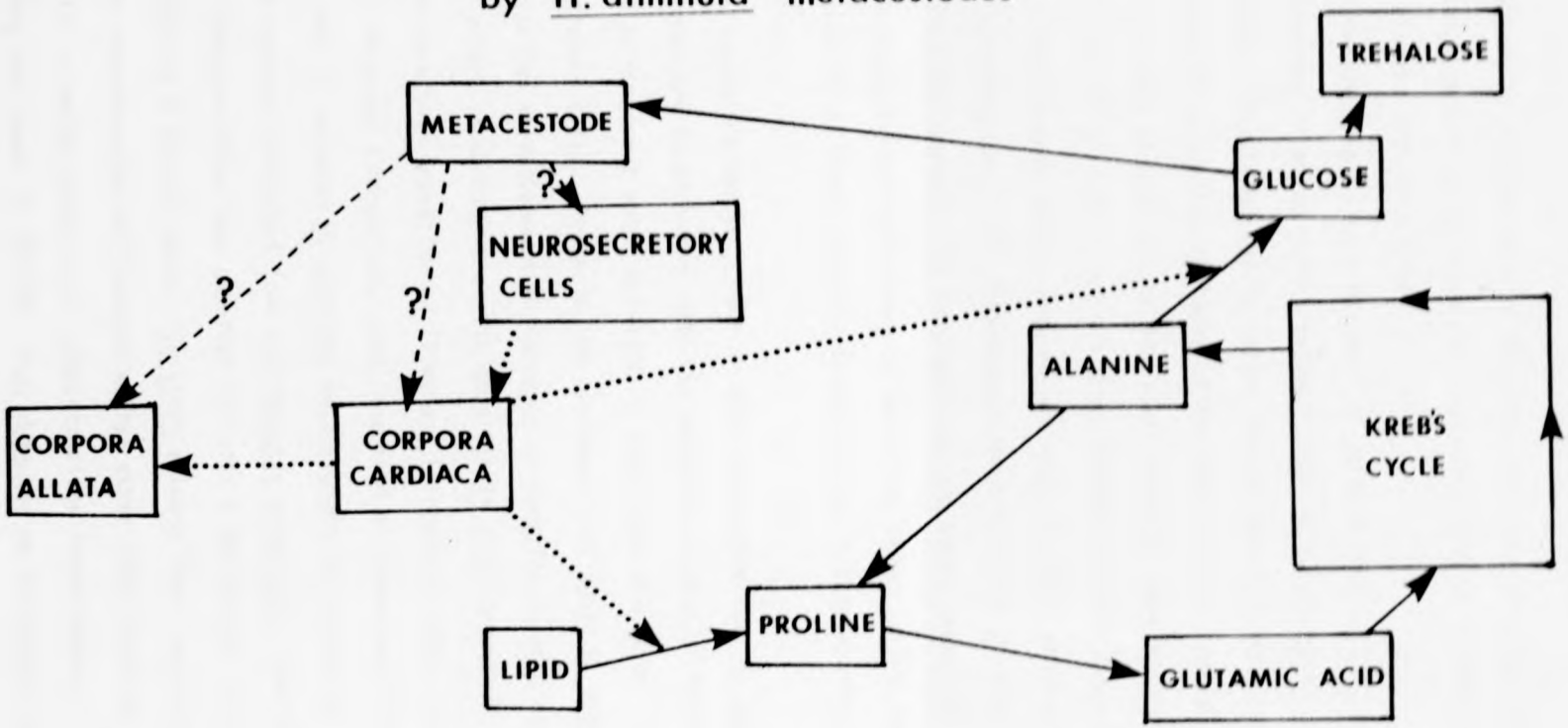
been discussed elsewhere. This supports the view that the first phase of vitellogenesis has already been primed and therefore cannot be affected by infection. Reduction in CA output and the consequent effects of this are thus only evident at a later stage, although the inhibitory affect is present throught the infection or at an early phase.

Data concerned with the egg-laying pattern in crowded beetles indicated that the effects of the decrease in vitellogenesis are relatively short lived, host recovery, in terms of ovulation, occurring by day 28. A comparision of fat body vitellogenin synthesis and ovary sequestration in non-infected and infected beetles was not carried out in beetles older than 15 days. An extension of these studies to encompass older infections would indicate whether events influenced by JH concentration are permanently altered or whether this is, indeed, a temporary effect. Observations concerning THSP concentration could also be explained in these terms. Vitellogenins did not continue to accumulate within the female haemolymph after the initial increase at 12 days-post infection. These findings might be expected if this increase occured as a pulse around this time. However, two alternative explanations present themselves; first, the rate of vitellogenin catabolism could increase in infected beetles thus preventing further accumulation in the haemolymph despite continued sequestration at reduced rates (this could be tested by an analysis of uric acid concentrations in faeces, fat body and haemolymph) secondly, an elevated vitellogenin concentration could produce a negative feedback effect causing the cessation of vitellogenin synthesis by the fat body. The occurrence of such a feed-back system, following the accumulation of vitellogenin resultant upon ovariectomy, has been postulated in several instances (for example, Borovsky, 1981 for the mosquito and

Harry & Applebaum 1982) for the locust).

The possible involvement of the Tenebrio endocrine system in the alteration of the concentration of individual amino acids in infected beetles was proposed in Ch. 10. Weeda (1981) reported that corpora cardiaca extracts injected into the Colorado beetle resulted in a decrease in alanine concentration and an increase in haemolymph glucose from 0.3-1.4 µg/µl 45 min after injection. Glucogenesis has also been investigated in T. molitor (Gourdoux, Laquellec, Moreau & Dutrieu, 1980 & 1983). Using three amino acids that are glucogenic in vertebrates, namely alanine, aspartic acid and glutamic acid, they found that all three were converted to carbohydrate, alanine being the most active; corpora cardiaca extracts were shown to increase this effect and an insulin-like molecule to diminish it. A decrease in glucose oxidation in T. molitor adults was observed in the presence of CC extracts by Gordoux (1980). He proposed that, under the influence of a presumed hormone in the extract, glucose was directed away from the pentose-phosphate cycle, which is normally active in the adult beetle. Proline synthesis was also found to be activated by a hormone from the corpora cardiaca (Weeda, 1981) in the Colorado beetle, and its synthesis in the fat body was subjected to feed-back inhibition. Both alanine and proline concentrations were significantly lowered in infected T. molitor ($p<0.001$) and it is interesting to speculate whether this effect was mediated via an alteration in corpora cardiaca activity. A hypothetical sequence of events leading to the observed decrease in these two amino acids and to the increase in glucose noted by Phillips (personal communication) is summarised in Fig. 11.1. Hormone-induced increase in proline synthesis could result in an increase in its conversion to glutamic acid and, via the scheme described in Ch. 10 (Fig. 10.1), an increase in alanine. Glucogenesis from alanine would produce increased

Fig.11.1 Possible mode of interference in amino acid metabolism and glucogenesis by H. diminuta metacestodes



concentrations of glucose and, if this step were the rate limiting one, it could result in the decreased concentrations of alanine and proline observed with infection.

It is interesting to note the work of Jeffs & Arme (1985a) on the characterization of amino acid transport sites in H. diminuta metacestodes. They found the K_t values for all amino acids investigated to be similar to those of the adult with one exception, proline. A value of 0.27 mM for the adult worm has been determined by Kilejian (1966), whereas Jeffs & Arme (1985a) obtained a K_t of 0.88mM. They proposed that a reduced affinity for this amino acid would be adventageous to the metacestode inhabiting, as it does, a proline-rich environment. If this were not the case, haemolymph proline concentrations could have an inhibitory effect on the uptake by the parasite of other possibly essential, but not so abundant, amino acids.

The suggestion has been made in this discussion that H. diminuta metacestodes are interferring with the endocrine system of their host by means of compounds which activate or inactivate glands or neurosecretory cells. There is some evidence that adult H. diminuta synthesise two stereoisomers of farnesol (a juvenile hormone mimic), and the effect of several prenoids upon the in vitro growth of the tapeworm have been investigated (Frayha & Fairbairn, 1969). Thorson, Digenis, Berntzen & Konyalian, 1968) reported an enhancement of growth rate of excysted H. diminuta metacestodes cultured with prenoid material extracted from Echinococcus granulosus hydatid cyst fluid. No such effect was observed by Tofts & Meerovitch (1974) investigating 6 day-old worms. In vitro culture for 6 days with various concentrations of farnesol methyl ether (FME) resulted in a reduction in weight gain at all concentrations investigated, including the lowest of 10^{-13}M. They detected no difference in

proglottis production and maturation when FME was added to the incubation medium, nor were ratios of protein:lipid:carbohydrate and dry weight altered. They did, however, detect a premature release of neurosecretory substance from the rostellar neurosecretory cells, and suggested that interference by FME in neurosecretion resulted in a decease in overall mass of strobila, although proglittization and maturation were unaffected. Fioravanti & MacInnis (1977) also found no growth-promoting effect when farnesol, farnesal or FME were added to the medium and, at high concentrations, these proved toxic. They concluded that these compounds may have very different effects, dependent upon the stage of the life cycle. The occurrence of ecdysteroids in cestodes has also been reported (for example, in Moniezia expansa (Mendis, Rees & Goodwin, 1984))

The role, if any that these hormones, or hormone mimics may play in the metacestode-beetle relationship is a matter for conjecture. The availability of techniques sensitive enough to detect juvenile hormones and ecdysteroids at physiological concentrations in both insect haemolymph and cestode tissue will enable this aspect to be investigated further. It is felt that a comparison of juvenile hormone concentrations in non-infected and infected beetles would enable the hypothesis that alterations in vitellogenin synthesis, secretion and sequestration in infected beetles are mediated via a reduction in this hormone, to be confirmed or rejected. Whatever the outcome of further research, it is clear that in common with other symbioses, the interrelationship between metacestode and host are precise, although the method of their orchestration remains to be discovered.

None of the findings presented in this thesis show unequivically that parasites interfere directly with the endocrine system of T. molitor However, as discussed above, aspects of reproduction and

also amino acid metabolism are under hormonal control. Decreased juvenile hormone concentration would result in the lowered rate of both vitellogenisis and vitellogenin synthesis and secretion noted in infected beetles, and it is felt that this affords the most likely explanation for the recorded observations. The proposition that there exists, in infected beetles, a potent corpora allata inhibitor, acting directly or indirectly on the gland or *via* the neurosecretory cells, is therefore submitted.

Ackowledgements

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* Not read in the original.

APPENDIX 1

Electrofocussing in thin layer polyacrylamide gels
Preparation of solutions

Fixing solution
100 g trichloroacetic acid (TCA)
10 g sulphosalicylic acid
Dissolved in 500 ml of distilled water and made up to 1000 ml with distilled water.

Washing solution
95% ethanol

Staining solution
1.5 g Coomassie Brilliant Blue G in 300 ml destaining solution
Stirred for 1 h at room temperature then filter.

Destaining solution
350 ml ethanol 95%
100 ml glacial acetic acid
Make up to 1000 ml with distilled water

APPENDIX 2

Electrofocussing in thin layer polyacrylamine gels
Preparation of gel solutions

Acrylamide solution, 29.1% (w/v)
29.1 g of acrylamide was dissolved in 75 ml of distilled water. The solution was stirred until clear, then made up to 100 ml with distilled water.
The solution was filtered and stored in a dark bottle at 4°C for a maximum of one week.

N,N'Methylenebisacrylamide (Bis), 0.9% (w/v)
0.9 g of Bis was dissolved in 100 ml of distilled water. It was filtered after cooling and stored in a dark bottle for a maximum of 1 week.

Ammonium persulphate, 1% (w/v)
1.0 g of ammonium persulphate was dissolved in 100 ml of distilled water.
This solution was used fresh each time.

APPENDIX 3

Electrofocussing in thin layer polyacrylamide gels
Preparation of stock solutions

Fixing solution
17.3 g sulphosalicylic acid
57.5 g trichloroacetic acid
Dissolved in 500 ml of distilled water.

Staining solution
0.460 g Coomassie Brilliant Blue G
Dissolved in 400 ml of destaining solution.

Destaining solution
500 ml ethanol
160 ml acetic acid
Mixed and made up to 2 litres with distilled water.

Preserving solution
500 ml destaining solution
50 ml glycerol
Glycerol was added to the destaining solution.

APPENDIX 4

SDS Polyacrylamide gel electrophoresis
Stock solutions

Phosphate buffer stock solution 0.2 M, pH 7.1
44.0 g $NaH_2PO_4 . H_2O$
258.0 g $Na_2HPO_4 . 12H_2O$
10.0 g SDS
Made up to 5000 ml with distilled water.

Electrode buffer
1 part buffer stock solution plus 1 part distilled water.

Acrylamide solution
22.2 g Acrylamide
0.6 g Bisacrylamide
Made up to 100 ml and filtered.
Stored in a dark bottle at 4° C.

Ammonium persulphate solution
150 mg Ammonium persulphate
Made up to 10.0 ml with distilled water.
Stored in a dark bottle at 4° C and made up freshly each week.

Bromophenol Blue
25 g Bromophenol blue
Made up to 10 ml with sample buffer.

Detergent solution
0.1 ml Triton X-100
Added to 100 ml of distilled water.

APPENDIX 5

Conventional polyacrylamide gel electrophoresis
Preparation of stock solutions

Tris-glycine buffer stock solution

75.1 g Glycine
2.5 g Sodium azide
Tris
The glycine and sodium azide were
dissolved in 3 litres of distilled water.
This solution was titrated with Tris to pH 8.9.
Made up to 5000 ml with distilled water.

Electrode buffer

5.0 ml Buffer stock solution
Made up to 100 ml with distilled water.

For all other solutions see Appendix 6 with the exception of the bromophenol blue which was made up in the Tris-glycine buffer.

APPENDIX 6

SDS Polyacrylamide gel electrophoresis
Preparation of solutions

Fixing solution
57.0 g Trichloroacetic acid
17.0 g Sulphosalicylic acid
150 ml Methanol
350 ml Distilled water
The acids were added to the mixture of methanol and distilled water.

Staining solution
1.25 g Coomassie Brilliant Blue G
227 ml Methanol
227 ml Distilled water
46 ml Glacial acetic acid
The dye was dissolved in the solution of methanol and distilled water.
The acetic acid was added and the solution filtered.

Destaining solution
1500 ml Ethanol
500 ml Acetic acid
Made up to 5000 ml with distilled water.

Preserving solution
300 ml Ethanol
100 ml Acetic acid
100 ml Glycerol
Made up to 1000 ml with distilled water.

All solutions were freshly prepared each month.

APPENDIX 7

The preparation of Tyrode's saline

NaCl	8.00g
KCl	0.20g
$CaCl_2$	0.20g
$MgCl_2$	0.10g
NaH_2PO_4	0.05g
$NaHCO_3$	1.00g
Glucose	1.00g

Made up to 1 litre with distilled water

APPENDIX 8

The preparation of KRT saline

KR saline

NaCl	70.20g
KCl	3.59g
$MgSO_4.7H_2O$	0.32g

Dissolved in 800 ml distilled water then add

$CaCl_2.6H_2O$	3.77g

Made up to 1 litre

KRT saline

1 volume KR saline

1 volume 0.25M Trismaleate buffer

8 volumes distilled water

APPENDIX 9

Buffer solutions for a six-sample, discrete dual-flow amino acid analyser

Sodium eluting buffers

To separate basic amino acids on a 15cm column

pH	4.56	5.9
Sodium concentration (N)	0.38	0.35
Sodium citrate $2H_2O$	186.2 g	171.6 g
Conc. HCl 35%	76.1 ml	32.5 ml
Octanoic acid	0.5 ml	0.5 ml
Brij, 35 50% soln.	10.0 ml	10.0 ml
Final volume with distilled water	5000 ml	5000 ml

Lithium eluting buffers

To separate acid and neutral amino acids on a 50cm column

pH	2.78	3.15	3.94
Lithium conc. (N)	0.25	0.30	0.30
Lithium citrate $4H_2O$	62.75 g	75.25 g	141.0 g
Lithium chloride	24.75 g	29.75 g	-
Octanoic acid	0.5 ml	0.5 ml	0.5 ml
Thiodiglycol	12.5 ml	12.5 ml	12.5 ml
Brij, 35 50% soln.	10.0 ml	10.0 ml	10.0 ml
Ethanol	250.0 ml	-	-
Final volume with distilled water	5000 ml	5000 ml	5000 ml

APPENDIX 10a

```
0015 DATA VAL,10,CYS1,10,MET,10,CYSTA,5,ILE,10,LEU,10,TYR,10,PHE,10
0005 DIM A$(32),A(32),N(32)
0010 PRINT TAB(10);"ANALYSIS OF AMINO ACID DATA"
0020 PRINT TAB(10);"----------------------------"
0030 PRINT
0035 PRINT
0040 PRINT "RUN NUMBER=";
0050 INPUT R
0060 PRINT
0070 FOR L=1 TO 6
0080 IF L>1 THEN 110
0090 PRINT "LOOP 1: STANDARD AMINO ACIDS"
0100 GOTO 130
0110 PRINT "LOOP ";L;": SAMPLE"
0120 GOTO 200
0130 PRINT
0140 PRINT "JOB NUMBER =";
0150 INPUT J
0160 PRINT
0166 PRINT
0170 PRINT "AMINO ACID";TAB(15);"CORRECTED AREA"
0180 PRINT
0190 FOR I = 1 TO 32
0200 READ A$(I),A(I)
0210 PRINT TAB(3);A$(I);TAB(19);
0220 INPUT C
0222 IF C>0 THEN 230
0224 LET C=1
0230 LET N(I)=C/A(I)
0240 NEXT I
0245 GOTO 500
0260 PRINT
0270 PRINT "NAME OF SAMPLE (2 IDENTIFIERS) = ";
0280 INPUT S1$
0290 PRINT TAB(33);
0300 INPUT S2$
0310 PRINT
0320 PRINT "JOB NUMBER = ";
0330 INPUT J
```

APPENDIX 10b

```
0332 PRI T "VOLUME OF SAMPLE (UL) = ";
0334 INPUT V
0336 PRINT
0340 PRINT "X-INT";TAB(9);"Y CORRECTED";TAB(20);"CONCENTRATION"
0360 PRINT
0365 READ F,A
0370 FOR I=1 TO 3
0380 PRINT X(I);TAB(9);
0390 INPUT Y
0400 LET C1=L/F
0410 LET F1 =C1/ (I)
0440 LET C2=C1*A/V
0450 PRINT TAB(21); 2
0460 IF I=3 THEN 470
0465 GOTO 420
0470 READ F,A
0480 PRINT
0490 NEXT I
0500 FOR J=1 TO 5
0510 PRINT
0520 NEXT J



0525 IF L=6 THEN 999
0530 NEXT L
0600 DATA ORN,10,LYS,10,ARG,10,HI,10,HIS,10,MET,10,CAM,10,ARG,10
0610 DATA PSER,5,TAU,5,PEIM,5,ASP,10,HYP,10,THR,10,SER,10,ASN,5,GLU,10
0611 DATA GLN,10,SARC,5,ALIP,2.5,PRO,10,GLY,10,ALA/CIT,12.5,AMA2,2.5
0620 DATA 1.000,3,0.997,2.5,0.999,3,1.001,2.5,0.993,3,1.001,2.5,0.991,3,0.994
0621 DATA 2.5,0.994,3,0.994,2.5
0999 END
```